ON TETHELIN AS A TISSUE-CULTURE MEDIUM[1]

by

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INTRODUCTION.

The growth-promoting substances which must be added to the coagulated plasma in order to enable tissues to proliferate *in vitro* for prolonged periods have been the subjects of a most intensive investigation for many years. Quite early in the history of tissue-culture technique it was shown that plasma and sera inhibit indefinite multiplication, the extracts of various tissues or embryos being essential for maintaining the growth and survival of tissues *in vitro* (4).

Of these nutritive media, embryonic extract has proved the most successful and apparently one of the most suitable growth-promoting substances, giving unretarded growth-rate for indefinite periods of time. Experiments have been made to isolate the actual growth-promoting elements of embryonic tissue-juice, but without success.

In the animal body the influence of growth-stimulating substances has long been known, and study of the lipoid secretions of the anterior lobe of the hypophysis has resulted in the isolation of three supposedly pure substances, namely, cholesterol, lecithin, and tethelin, which are active growth-stimulators. Tethelin, one of these substances isolated by Robertson (11), is the subject of this present investigation.

While it is known that tethelin increases the growth-rate of carcinoma in rats (12, 13), and promotes marked acceleration in the tissue repair of wounds, it has not apparently been used as a culture medium for *in vitro* cultures. Barney (3) has given clinical evidence of the efficiency of tethelin in the treatment of chronic ulcers, this substance appearing to have caused healing where other recognized treatment has failed.

It is the purpose of this investigation to observe the effects of tethelin on embryonic epithelium when cultured *in vitro* in a medium of plasma containing a given saline solution of tethelin. The growth-rate in this medium has been compared with controls made in a medium of plasma and chick embryo extract.

[1] This work was carried out under the control of the Cancer Research Committee of the University of Sydney and with the aid of the Cancer Research and Treatment Fund.

TECHNIQUE.

The glandular wall of the intestine was removed, under sterile conditions, from a seven-day chick embryo, and cut with sharp iridectomy knives into very small-sized fragments, which were washed free of blood and other foreign tissues by several drops of warm physiological saline of pH 7·5.

Plasma, obtained from the ventricles of adolescent chickens, was stored in paraffin-coated Wasserman tubes, cooled at 0°C., and employed as a supporting medium. The cultures were made on sterile No. 1 cover-slips, on which was placed a small drop of plasma of constant volume. The tissue fragments were then transferred to the plasma, and great care was taken to imbed the tissue within the plasma to allow subsequent growth near the under-surface of the cover-slip.

The following media were then added in equal volumes to each of the cultures:

(A) A solution of 100 milligrams of tethelin in 25 cc. of sterile normal saline of pH 7·5.

(B) Equal volumes of embryonic extract and a solution of 100 milligrams of tethelin in 50 cc. of sterile normal saline of the same pH.

(C) Embryonic extract made from seven-day chick embryos.

The cultures, after 28 hours' incubation at 38·5°C., were fixed in osmic vapour and stained with Heidenhain's iron haematoxylin.

General Appearance of the Cultures in vitro.

When cultures of intestinal epithelium in a medium containing either tethelin alone, or tethelin and embryonic extract, are compared with control cultures made with embryonic extract, a vast difference in the growth-rates is at once apparent (figures 1 and 4). The explants containing tethelin alone showed more extensive proliferation in some instances than those containing embryonic extract and tethelin, but the differences in the growth-rates of these two types of cultures was not as pronounced as in the case of controls containing embryonic extract.

In cultures containing tethelin a membrane is quickly formed even with only 28 hours' incubation, whereas in the control cultures the cells were observed to migrate in tongue-like processes, and only with further incubation did they form continuous membrane (figure 9).

The morphology of the epithelial cells was observed to vary according to the types of membrane formed, the latter being dependent upon the density of the medium, as suggested by Uhlenhuth (15, 16), and later confirmed by Fischer (8).

It must be emphasized that in every instance the explanted tissue was imbedded within the plasma clot, and not resting on its surface, according to

the technique of Ebeling and Fischer (7). In order to use critical illumination, and also to obtain good microphotographs, it is essential that the cells under observation should be as close as possible to the under-surface of the cover-slip, rather than at the deeper levels in the medium or on the surface of the clot.

The cells in the tethelin cultures are typically polygonal in outline, where the membrane is homogeneous, and closely aggregated to give a pavement-like formation (figures 5 and 7). The nuclei are rounded and rarely oval, contrasting markedly with the preparations in embryonic extract; the cytomicrosomes, particularly the mitochondria, are very abundant. In many instances the mitochondria are closely packed around the nuclear membrane, presumably the result, as one of us has demonstrated (9), of a surface-tension phenomenon.

Where more pronounced liquefaction, and the consequent sparseness of the fibrin strands, occurs in the medium, the epithelial cells are elongate, with a single large pseudopodium, orientated generally in the direction of migration (figure 8). The spindle-shaped cells have almost spherical nuclei, and the mitochondria may be observed within the pseudopodia as well as aggregated in groups around the nuclear membrane.

Generally on the extreme periphery of the cultures, or bordering areas of extensive liquefaction, isolated cells may be studied (figure 6). In these the spherical nuclei are relatively reduced in size, lying within a much more vacuolated cytoplasm. Large fat globules are common within these cells.

Cultures grown in embryonic extract give a superficial appearance after 28 hours' incubation of ordinary fibroblasts (figure 4). The outgrowths are attenuated, and do not coalesce to form a membrane for some time after explantation.

The individual cells, by virtue of their compactness, appear somewhat elongated, rather than polygonal or pavement-like, as in the tethelin preparations (figures 2, 3, and 9). When membrane formation has commenced, adjacent cells of different outgrowths appear to form elongate pseudopodia, and eventually give rise to a continuous membrane. The nuclei of these cells are invariably oval in contour (figure 3), and the chromatin bodies in our preparations were always very clearly defined (figure 2). Apart from these details, however, the cytomicrosomes of cultures in embryonic extract were not so well defined as in similar preparations made in a medium containing tethelin.

Tethelin appears to stimulate the growth of intestinal epithelium *in vitro*, so that extensive homogeneous membranes are formed after 28 hours' incubation, and the individual cells in these cultures may be studied to much greater advantage from a cytological standpoint than in similar explants grown in embryonic extract.

DISCUSSION.

The culture of epithelium *in vitro* has been extensively investigated since the commencement of tissue-culture technique, but unlike the connective and

other tissue elements it has presented several difficulties. Actually the cultivation of a continuous strain of epithelium, preserving the sheet-like formation and the normal characteristics of the individual cells, was not successful until recently, when Ebeling and Fischer (7, 8) discovered the required technique.

It is agreed almost unanimously that epithelia tend to show, under normal methods of explantation, a period of latency before any marked proliferation occurs. The initial growth in many instances is also quite irregular. These observations have been confirmed in the present investigation, and the difficulties cited above appear to be minimized by the use of tethelin instead of embryonic extract. This growth-stimulating lipoid so markedly increases the initial growth-rate that an extensive membrane is formed after only 28 hours' incubation. This membrane, which is an unbroken one when the medium has not been too intensively liquefied, contrasts most definitely with the small tongue-like migrations of cells observed after a similar period of incubation in embryonic extract.

Uhlenhuth, in his early communications (15, 16), has recorded these two types of membrane formation, observing that in a softened, more fluid medium the membranes are more homogeneous, whereas in a firmer medium containing less fluid the typical tongue-like outgrowths occurred. With further incubation more liquefaction of the plasma took place, causing the cells to separate somewhat, and the membranes finally to disintegrate. An exceedingly liquid medium led to isolation of the individual cells, and finally with the increased surface tension acting on the isolated cell walls they assumed a spherical contour.

From the researches of Carrel and Baker (6) it is now evident that the chemical substances from which cells synthesize new protoplasm are constituents of embryonic juice, and not of blood serum. Many attempts have been made to determine the nature of these substances used by tissues, and to isolate the principle responsible for growth. Meanwhile Carrel has given the general name of Trephones to the growth-promoting substances. In the case of embryonic extract the trephone appears to be a protein of a very unstable nature (5), and Schazillo (14) has shown that similar growth-promoting substances may be found in lymphocyte extracts and in peritoneal exudates.

Using the pepsin hydrolytic products of pure proteins, such as crystalline egg albumen and edestin, Baker and Carrel (1) succeeded in stimulating the growth of fibroblasts for a certain time *in vitro*, but they failed to obtain continuous undiminished proliferation, as occurs with embryonic extract. With similar digests of fibrin some remarkable growths have been observed by these investigators (5), even exceeding those of controls in embryonic extract, when the digestion of the fibrin was carried to an optimum degree.

In experiments *in vivo* the growth-stimulating nature of the lipoid tethelin has been demonstrated both in normal animals and in rats bearing transplantable neoplasms. Robertson (10) has shown that tethelin accelerates grown in normal,

post-adolescent animals, and that hypodermic administration of tethelin causes increased proliferation and metastasis in rat carcinomata.

The action of these lipoids appears to be a closely specific one, for other alcohol-soluble extractives of the anterior lobe of the pituitary may either exert no appreciable effect or cause retardation of malignant tumours. The effect of tethelin as a growth-promoting substance has been fully demonstrated in the present work dealing with *in vitro* cultures of normal embryonic tissues. Carrel and Baker (2), however, have concluded that the rates of growth of sarcomatous fibroblasts in peptic digests of the anterior lobe of the pituitary and of controls in embryonic juices were approximately equal. It is suggested that the separation of the growth-promoting elements as tethelin has given the increased proliferation in our cultures, whereas the possible inclusion of growth-inhibiting substances, already proved to be present in pituitary extracts, has resulted in the negative conclusions of Baker and Carrel.

SUMMARY.

1. The growth-promoting qualities of tethelin have been confirmed *in vitro*.

2. Cultures of intestinal epithelium from a seven-day embryo chicken in a medium of chick plasma, embryonic extract and tethelin or chick plasma and tethelin, give more extensive proliferation than similar cultures in plasma and embryonic extract; membrane formation taking place after 28 hours' incubation in the former but not in the latter cultures.

The authors wish to express their indebtedness to Professor Brailsford Robertson, of the Adelaide University, for his kindness in supplying the tethelin, and also for his helpful interest during this investigation.

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EXPLANATION OF PLATES.

All microphotographs were taken of cultures after 28 hours' growth in vitro, in a medium of embryonic extract or a tethelin mixture, after fixation in osmic vapour and afterwards stained by the Heidenhain iron haematoxylin process.

Plate 1.

Figure 1. Microphotograph of portion of the periphery of an explant cultivated in plasma and tethelin, showing extensive membrane formation after 28 hours' incubation. Observe the healthy condition of the cells.

Figure 2. Showing isolated tongue-like outgrowths of a similar culture grown in embryonic extract. Note the apparent nucleolus undergoing division.

Plate 2.

Figure 3. Enlarged microphotograph of portion of a control culture grown in embryonic extract, showing the commencement of membrane formation and cytological details in the epithelial cells.

Figure 4. A portion of a periphery of a control culture taken with a lower magnification. Compare the extent of proliferation and the absence of membrane formation with that of Figure 1.

Plates 3 and 4.

Figures 5 and 7. Enlarged microphotographs depicting the epithelial membrane as seen in a culture in a tethelin medium. Note the rounded condition of the nuclei and the position of the mitochondria, as well as the morphology of the cells.

Figure 6. Isolated rounded epithelial cells at the extreme edge of the membrane shown at greater magnification. Observe the large lipoidal droplets together with the mitochondria. The nucleus is deeply stained.

Figure 8. Elongated epithelial cells in the regions where the medium has liquefied, showing the discontinuous membranes and also the peculiar structure of the cells.

Plate 5.

Figure 9. Enlarged microphotograph of the peripheral region of a control culture grown in embryonic extract. Observe the tongue-like outgrowth and the commencement of membrane formation.

Fig. 1.

Fig. 2.

PLATE 1.

Fig. 3.

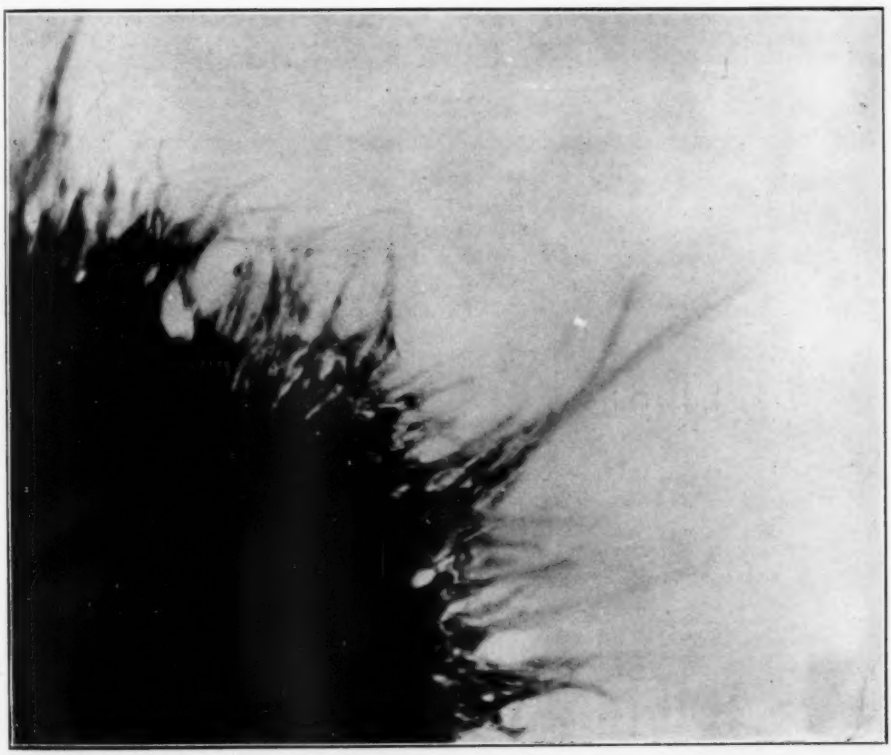

Fig. 4.

PLATE 2.

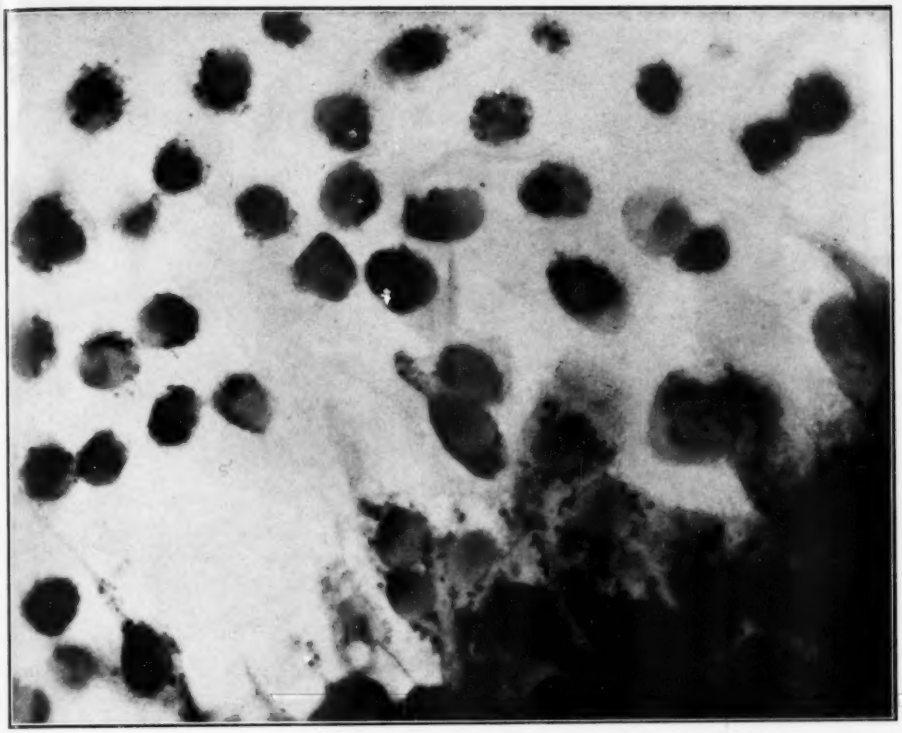

Fig. 5.

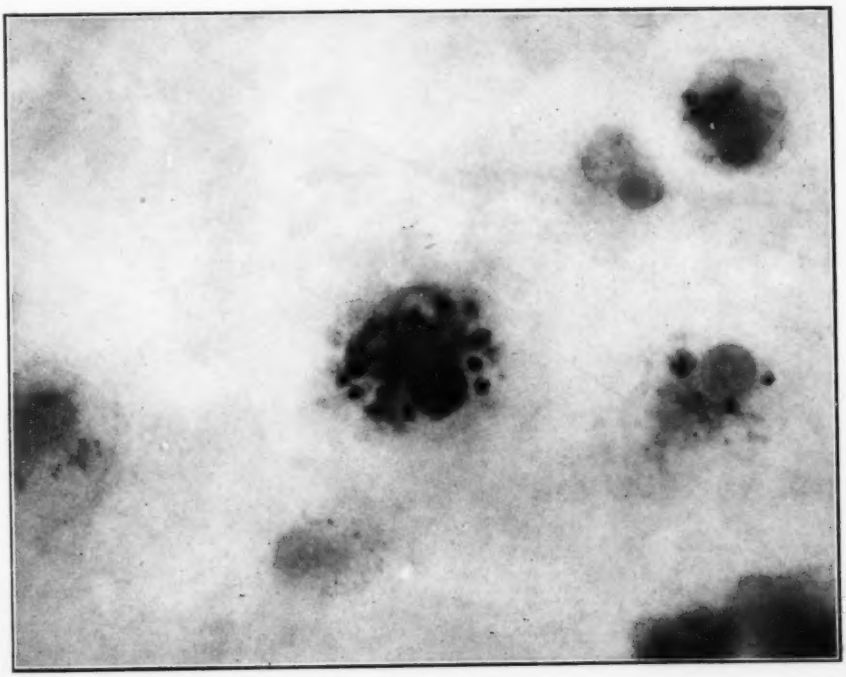

Fig. 6.

PLATE 3.

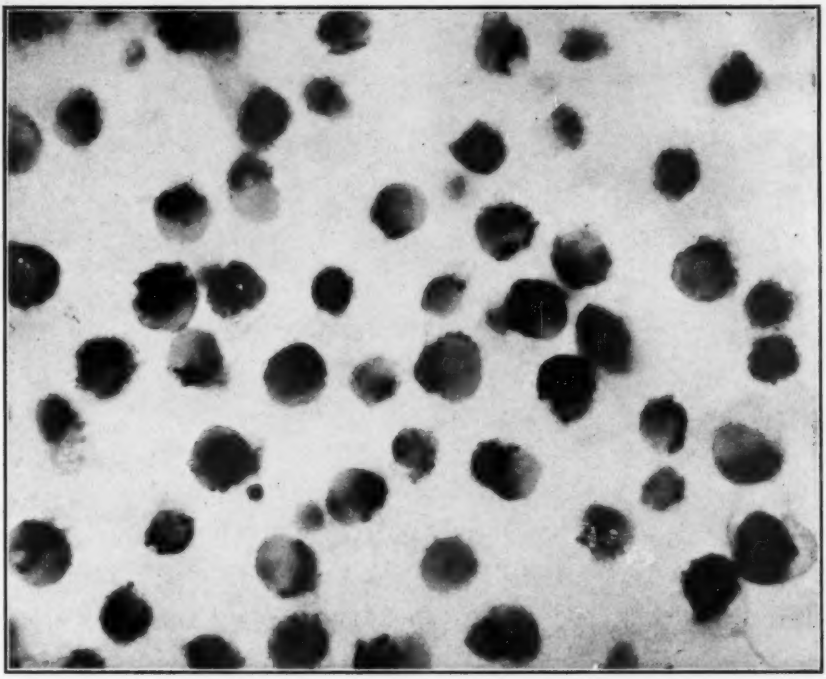

Fig. 7.

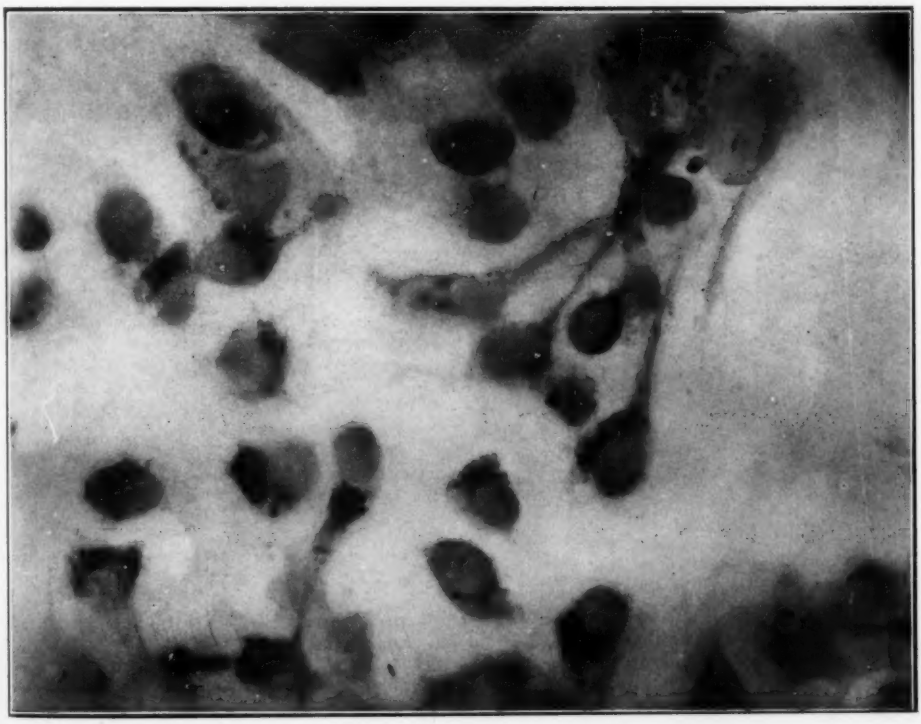

Fig. 8.

PLATE 4.

Fig. 9.

PLATE 5.

CYTOLOGICAL OBSERVATIONS OF A LUMSDEN RAT SARCOMA *IN VITRO*[1]

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INTRODUCTION.

Recently the appearances of various strains of sarcomata when grown *in vitro* have been described by several investigators (4, 5, 6, 8, 9, 13, 14, 16), but, nevertheless, a great deal of controversy still exists concerning the nature, structure, and behaviour of the cell-types composing these cultures. The object of this present communication is to describe the general cytology, behaviour, and manner of growth of a Lumsden rat sarcoma when cultivated *in vitro*, with a view to obtaining some new data, which may help to throw further light on these matters.

The authors wish to take this opportunity of acknowledging their indebtedness to Dr. Lumsden, of the Lister Institute, London, for providing the original strain of rat sarcoma, and also to Professor C. Witherington Stump for his kind interest and help during this investigation.

Material and Technique for Tissue Cultivation.

Throughout this investigation a Lumsden rat sarcoma was employed. The cultures, which were prepared by the usual hanging-drop method, were grown *in vitro* together with chicken or rat plasma.

When obtaining chick plasma the blood was collected by means of dry, sterile canulae from the ventricle, and was allowed to flow into paraffin-coated Wasserman tubes previously cooled to 0°C. The supernatant fluid obtained after centrifuging was pipetted off and used as the supporting medium. The rat plasma was obtained in a similar manner; but, owing to the facility this type of plasma has for clotting, it was found advisable to collect the blood with frozen, paraffined canulae. The blood was taken from the ventricle of the rodent, and in order to prevent excessive bleeding from the intercostals a small incision was

1 This work was carried out under the control of the Cancer Research Committee of the University of Sydney and with the aid of the Cancer Research and Treatment Fund.

made between the 6th and 7th ribs, through which the canula was thrust into the heart. By employing this method the necessary amount of blood was collected without much difficulty. The plasma thus obtained was kept in paraffined tubes, stored in a refrigerator, and used when necessary.

Throughout these operations adolescent animals were used, which had been previously starved for 24 hours.

When chicken embryonic tissue juices were needed, 7 days' old embryos were placed into sterile Petri dishes, where all traces of blood were removed by bathing the tissues in physiological saline solution having a pH 7·5. The given tissues were then placed into another sterile dish, and with the aid of a pair of de Wecker's iris scissors were minced to a pulp. The mixture, after standing a short while, was then centrifuged for several minutes, the supernatant fluid was pipetted off, and then used as the extract.

When selecting pieces of neoplastic tissues about to be cultivated *in vitro*, tumour fragments were obtained that were comparatively free from the normal tissues of the host. Small fragments were then removed to a sterile cover-glass, where equal volumes of plasma and embryonic extract were added with sterile pipettes. The fluids were then mixed together with the aid of a cataract knife. The preparations about to be cultivated were then inverted over the hollow ground slide, sealed in the usual manner, and then incubated at 38·5°C.

In order to carry out investigations *in vivo*, the explants were placed upon a warm Hearson stage kept at body temperature.

Fixed preparations were obtained by first immersing the cultures for 20 minutes in a warm Ringer's solution, in order to prevent coagulation of the plasma. The explants were either treated by osmic vapour or fixed in a solution of 2% formalin in saline, and then stained with Heidenhain's iron haematoxylin.

In order to record the appearance, as well as the internal structure, of the living cells, a series of microphotographs were taken at various intervals of growth. In some cases it was found necessary to wash the explants in saline in order to reduce the watery film between the cover slip and the slide sufficiently to allow critical illumination of the living cells, and in order to avoid distortion of the cell types due to pressures, the cover-glass containing the culture was again sealed to a concavity slide. Special attention was paid to the centring of the objective and the adjustment of the source of illumination.

General Characteristics and Appearance of Growth in vitro.

When a healthy, actively-growing culture, after 7–9 hours' incubation *in vitro*, is examined under the microscope many cells may be detected migrating from the explanted tissue in a radial manner along the cover-slip, or else upon the surface membrane of the hanging-drop.

Further, observations upon a 24 hours' old culture will show that the original tumour fragment has become surrounded by a broad band of cells, and

it is at this stage that the general characteristics and appearance of the growth may be studied to advantage.

The cells composing this zone of new growth have a slightly opaque appearance, and owing to their isolated condition, their extremely irregular outlines, as well as their internal structure, may be observed quite clearly.

Several authors have shown quite conclusively that the area of new growth surrounding the explanted tissue is dependent upon the culture medium employed; and recently Walton (24), who investigated the rate of growth of mammalian tissues *in vitro* in different extracts, demonstrated that they are specific in their action upon the growth of the cells, some being inhibited by one extract and stimulated by another.

And, similarly, we have shown that the appearance of the cultures depends greatly upon whether they are cultivated in a medium containing rat or chick plasma. The tissues grown in the former medium together with embryonic extract have a more compact appearance than those cultivated in a medium containing the latter. The total growth is much greater in the former than in the latter, and the cells composing the general cultures possess a more healthy appearance.

A more detailed examination under the higher powers of the microscope of the cellular elements composing the periphery of actively growing cultures reveals three cell-types. They are so strikingly different from each other in both their behaviour and morphology as to attract immediate attention.

The first type, which greatly predominates in numbers in all our cultures, corresponds to the fibroblast-like elements already described by several authors (4, 8, 9, 16). The second type represents the sarcoma cells proper, and the third type (highly amoeboid in nature) possesses a similar appearance to the clasmatocytes first described by Lewis and Gey (16). In all actively growing cultures of sacromata observed these three cell-types were a specific feature, and the object of the succeeding chapters will be to discuss in greater detail the morphological structure and behaviour, as well as the general cytology of these cellular elements, so characteristic of the sarcomata when cultivated *in vitro*.

General Morphology of Cell-types.

In order to observe the morphology of the cellular elements composing these cultures, observations were made upon living unstained and fixed preparations of cells composing the periphery of the explant after a 28 hours' growth *in vitro*. The cells growing in this region are more isolated, thereby allowing their morphological characteristics to be studied to advantage.

Examination of Preparations Fixed after 28 Hours' Growth in vitro.

The fibroblast-like elements, which in all our cultures greatly predominate in numbers, vary in size and form, but all the fully-grown cells are elongated,

and appear to be bifurcated in the direction in which they happen to be migrating (see figure 12). A detailed and lengthy examination of these cell-types shows this phenomenon to be a specific morphological character common to all the fully-developed fibroblast-like elements composing the L.R.S. The long protoplasmic strands sent out by these cells very often intermingle with each other in such a manner as to give the appearance of a cytoplasmic network. The nuclei of the young pseudo-fibroblasts are generally spherical, while those of the older cells are elongated. In both cases the nuclei are densely packed with large chromatin bodies. The protoplasm in fixed preparations has a foam-like appearance, which may be due to the fixation.

The sarcoma cells within the explants appear to be either spherical or oval in outline; the majority, however, seem to be of the former structure. The nuclei, which are densely packed with chromatin, are large and round, and generally lie to one side of the cell. A more detailed morphological examination of the components around the periphery of the cultures shows that those cells which have migrated farthest out into the plasma clot possess a slightly more elongated appearance, and thus come to resemble the young fibroblast-like elements in outline. The possibility that the pseudo-fibroblasts are merely morphological variations of the tumour cell, and not generically separate types, immediately presents itself, and will be the subject of a discussion in a later portion of this communication.

Cells similar to clasmatocytes have been described by several authors (5, 16) as being characteristic components of the sarcomata when grown *in vitro*. These wandering cells, which appear in smaller numbers in all our cultures, present a striking contrast when compared with the pseudo-fibroblasts and sarcoma cells. (See figure 15.) They appear more active in their movements, judging by the number of pseudopodia which appear to be thrown out simultaneously in a very diverse manner, thereby giving them an extremely irregular outline. Their nuclei, which contain numerous large chromatin granules, are large, and generally distorted, and often project into the pseudopodia. The general protoplasm of these cells presents a highly vacuolated appearance. The wandering cells vary considerably in size, and all are characterized by their extreme irregularity in shape, as well as their active movements.

Examination in vivo of 28 hours' Cultures by Direct Illumination.

In order to observe the general morphology and behaviour of the living cells during growth and differentiation, the direct illumination method was employed. Throughout this investigation the explants were placed upon a warm stage maintained at body temperature. By this means the outlines of the cells, together with their internal structure, are shown quite clearly. The general protoplasm appears clear, transparent, and homogeneous. (See figures 2, 3, 4.) The nuclei, as well as dull, greyish bodies, the chromatin masses,

are distinctly seen, together with a variable number of highly refractive cytomicrosomes, which are continuously undergoing active irregular movement within the general protoplasm of the living cells.

For this study the isolated cells forming the periphery of the growing cultures were chosen, as in this region the plasma clot is generally sufficiently thin to allow critical illumination of the cells.

When the living cultures are examined under the lower powers of the microscope the three distinct cell-types, together with several intermediate forms, may be seen; this field presents a similar appearance to those seen in fixed preparations. Of late several workers, when examining fixed and stained cultures, have suggested that the transitional cell-types were probably due to cell shrinkage caused by faulty fixing and staining.

Observations of a fully-developed pseudo-fibroblast under an oil-immersion lens shows the cells to be slightly more elongated in the direction in which they happen to be migrating, and bifurcated, finger-like processes may very often be seeen adhering to the cover-glass.

The sarcoma-cells present a similar picture to those seen in the fixed cultures, but those present in the peripheral zone tend to adopt a more elongated structure. Their large nuclei, which tend to lie to one side of the cell (see figures 2, 6, 4), together with the nuclear membrane and chromatin bodies, are distinctly seen. When examining these cells in more detail in fixed preparations their outline appears sharply defined, while observations *in vivo* show delicate little radial protoplasmic processes, which are sent out from the body of the tumour cells, very often giving these components a star-like appearance.

The cells having the appearance of clasmatocytes, when observed in the living state, may easily be distinguished from the other two cell-types by their general irregularity in outline, which is due to the many pseudopodia which are continuously being thrown out and withdrawn. Their distorted nuclei, which project into the pseudopodia, are clearly seen.

The morphology of these cells, when seen *in vivo*, compares favourably with those observed in fixed cultures. They appear less numerous than any of the other cell-types, and owing to their highly amoeboid structure migrate the greatest distance from the original explanted tissues. An examination of preparations fixed and stained after 28 hours' cultivation *in vitro* showed many intermediate forms between the round sarcoma cell and the pseudo-fibroblasts, and the possibility then suggested itself that the fibroblast-like elements were merely morphological variations of the malignant cell, and not generically separate types. As this investigation *in vivo* affords an excellent opportunity to decide upon the nature and origin of these cells, more detailed observations were made, and the appearance of these living cells during the transitional stage is recorded by a series of microphotographs (see figures 3, 4, 9). Recently

while studying the growth characteristics of a mouse sarcoma *in vitro* (37 C) (8), a similar phenomenon was observed, but owing to technical difficulties we were unable to record this effect by means of photography.

The round malignant sarcoma cell that is about to transform into a pseudo-fibroblast gradually becomes fusiform by sending out finger-like protoplasmic processes from one or either end, which generally adhere to the surface of the cover-glass. At intervals these pseudopodia are withdrawn, and fresh processes are again sent out, and, as the body of the cell becomes more elongated, the pseudopodia increase in thickness and in length, and very often reach across the field, as an examination of the microphotographs on plates 3 and 4 will depict.

Thus gradually the tumour cell comes to assume the structure and appearance of the fibroblast element, so characteristic of the sarcomata when cultivated *in vitro*. Observations of the nucleus during the transitional phase of the malignant cell are of interest. As the round monocyte, together with its large spherical nucleus, slowly lengthens out, the nucleus gradually becomes fusiform, and by the time differentiation has occurred the nucleus of the newly-formed pseudo-fibroblast has become elongated in shape. The results of these investigations upon the behaviour of these living malignant cells *in vivo* strongly suggest that a histogenetical relationship exists between these two cell-types.

Cytology of Cultures.

Throughout this investigation the general cytology of the cultures was carried out upon living cells, with the aid of a direct illumination. The cytology of the cytoplasm is best studied in explants that have been cultivated in rat plasma and embryonic extract and in those cells that adhere to the cover-glass in the periphery of the new growth, as in this region the plasma clot appears thinner.

It is hardly necessary to stress the advantages of tissue culture as a cytological technique. By employing the direct method the internal structure of the isolated individual cells appears sharply defined, the general protoplasm is clear, transparent, and homogeneous, and the nuclei, together with the chromatin bodies, which appear within the nuclei as dull, greyish bodies, may be seen quite distinctly (see figures 1, 4, 9). As seen with an oil-immersion lens the various highly refractive cytomicrosomes present a very striking field. The most conspicuous and the most brilliant of the cell components are the mitochondria (see figures 4, 5). These bodies, as seen by direct light, appear to be exceptionally brilliant, and may be observed in actively growing cultures undergoing irregular movement within the clear, transparent protoplasm of the living cells.

That the mobility of these granules is dependent largely upon the temperature at which the cultures are growing is easily demonstrated. As soon as the temperature is lowered they become less active, and immediately the temperature is raised the rapidity of their movements increases.

In order to record the appearances of these cytomicrosomes within the living cells by means of microphotography it was necessary to slacken the activity of these cell inclusions by lowering the temperature considerably.

The mitochondria within the living cells appear to be scattered somewhat irregularly throughout the protoplasm, but they are generally seen to occur in larger numbers around the nucleus and the periphery of the cells.

In previous papers (10, 11) one of us has pointed out that this effect, such as is seen in many cells, is a surface-tension phenomenon apparently dependent upon the phosphatidal nature of these bodies. The movements of these brilliantly refractive bodies are most fascinating to watch. Occasionally a rod-shaped mitochondrium may come to rest, constrict in the middle, until finally two small, spherical bodies are formed, which in their turn elongate, and give rise finally to the original rod-like structure.

The appearance and structure of these bodies within the living cells is also largely dependent upon the temperature at which the explanted tissues are growing; when the temperature is lowered these cell components gradually begin to round off, and by degrees assume the spherical condition (see figure 10). Mitochondria are more numerous in the fully grown fibroblast-like elements than in the clasmatocytes, although in the latter type of cell they appear to be undergoing more violent movements, owing to the continuous internal turmoil of the cells.

Still more interesting is the behaviour of the cell inclusions within the pseudopodia of the tumour cells during the transitional phase. The cells send out long pseudopodia, which occasionally stretch right across the field of vision (see figures 5, 7, 8).

Suddenly little bubble-like bodies containing mitochondria and other droplets proliferate and separate from the tip of the pseudopodium. These little bubble-like processes migrate a small distance from the cell, then finally swell up and burst, thereby liberating the mitochondria and other cell constituents into the surrounding medium (see figures 1, 4, 9); later the pseudopodium is slowly withdrawn.

Recently the behaviour and function of mitochondria have been the subject of much study by cytologists and biochemists (12, 18, 21), and they concur that the behaviour of these bodies strongly suggests that they are of an enzymatic nature.

The lipoidal droplets are very bright and conspicuous, and present a most brilliant field. They appear to occur in larger numbers in the clasmatocytes than in any of the other cellular elements, and are less active in their movements than the mitochondria. They vary greatly in size, and move about in little groups within the protoplasm of the living cells, and have a tendency to accumulate at one side of the nucleus. The movements of these droplets depend upon the activity of the cells; occasionally little groups of these refractive

globules are caught up by a protoplasmic current, separate out, and thus become distributed throughout the cell cytoplasm. During pseudopodial activity only the small droplets appear to be swept into the pseudopodium, while the larger ones remain within the body of the cell. These lipoidal droplets appear more numerous in old cultures or in those in which the temperature has been lowered (see figure 10), and for these reasons some authors have termed them "degeneration granules." Lambert and Hanes, who have observed also the increase in numbers of these bodies with the age of the cell, do not regard the presence of the cell-components as evidence of degeneration, as has been frequently suggested, but regard their presence as indicative of active anabolic function on the part of the cell, and they further suggest that they are synthesized by the cell from the soluble elements of neutral fat present in the plasma itself. In view of this interpretation it is of interest to quote the recent work of Cowdry and Scott (2, 22), who content that the cell mitochondria may, under certain conditions, transform into droplets with pronounced fatty and lipoidal properties.

While observing several actively growing cells round the peripheral regions of the cultures by means of direct illumination, certain intracellular structures having the appearance of a Golgi apparatus were detected lying in the cytoplasm of the living cell and in close relation to the nuclear membrane. The appearance of these reticular bodies was recorded by microphotography (see figure 6).

The general arrangement of the so-called Golgi apparatus is similar in appearance to the Golgi reticulum as seen in fixed preparations in many vertebrate cells.

Recently Strangeways and Canti (23), while studying the cytological constituents of certain cells *in vivo* by means of a dark-ground illumination, were unable to depict any structure which resembled the Golgi apparatus, but Fauré-Fremiet (7), with a dark-field condenser, saw rodlets in öocytes of Tegenaria, which he considered to be Golgi material; and Rau, Brambell, and Gatenby (20) also succeeded in depicting the dictyosomes of the Golgi apparatus within the living Helix spermatocyte cell, previously stained *intra vitam* with janus green, employing the iodine method of Lewis. Of late the reality of these bodies has been contested by the claims of Parat, Walker, and Allen (25), who contend that the Golgi apparatus is a mythical substance, perhaps an artefact due to faulty technique. The majority of investigators have been unable to stain *intra vitam*, but more recently Ludford (17), Wen-Chao Ma, and others workers (26, 27) claim to have stained Golgi material *intra vitam* with trypan blue, neutral red, and Sudan III.

Apart from whether these cytomicrosomes can be stained successfully *intra vitam* or not, preparations of fixed material demonstrate clearly that the Golgi substance exhibits a definite behaviour during mitosis, and based on these considerations alone their conclusions seem hardly justified.

At the present moment very little is known concerning the physiology of the Golgi elements. Nevertheless, it is of interest to note the recent work of Bowen, Ludford, and Nassanov (1, 15, 19), who contend that these intracellular structures play a very important rôle in secretory processes.

DISCUSSION.

A résumé of the recent literature upon the cultivation of sarcomata *in vitro* shows that a great deal of controversy exists concerning the cytological structures and behaviour of the cells composing the general growth of the cultures; and therefore we have taken this opportunity of reinvestigating this interesting field, with the idea that some fresh conclusive data might be obtained.

Before discussing our observations it is well to point out how few authors have availed themselves of the opportunity of observing the behaviour of these cells *in vivo;* most of their conclusions having been based on preparations fixed after various periods of growth *in vitro.* During this present investigation most of our results were directly acquired from the study of the appearance and general behaviour of the living cells as shown by direct illumination.

Lewis and Gey (16), when observing a mouse sarcoma *in vitro,* described two distinct cellular elements migrating from the explanted tissue; the first type corresponding to the cells detected in our cultures as those having the characteristic appearance of clasmatocytes; the second type representing the sarcoma cells proper. Lambert and Hanes (13), for example, recognized, in fixed cultures of mouse and rat sarcomata, two types of cells: a long, spindle-shaped cell (which corresponds to the pseudo-fibroblasts in our own cultures), and a second type which, in actively growing cultures, is characterized by its extreme irregularity and shape, caused by numerous pseudopodia, which protrude from the cell body in a bizarre manner. These authors further conclude that the two cell-types are not generically separate, but merely structural variations of each other.

Fell and Andrews, who recently made a cytological study *in vitro* of the Jensen rat sarcoma, depict in their cultures two separate cell-types, those resembling fibroblasts and cells having the appearance of wandering cells. They further hold that these cells occur in about equal numbers in all their preparations. The latter type represents the clasmatocyte of Lewis and Gey. In concluding, these authors suggest that the clasmatocytes might be the malignant element of the rat sarcoma, a contention with which Carrel (3) is not in accord.

When critically examining these two cell types in fixed cultures, by means of direct illumination, no transitional or intermediate structures were observed which would suggest any such phenomenon. Fell and Andrews further suggest that the pseudo-fibroblasts represent the stromal element. The question therefore naturally arises as to the character and origin of these two cell-types. When examining paraffin sections of sarcomatous tissue it is practically impos-

sible to detect these highly amoeboid structures, and they are so closely packed together as to obliterate their cytoplasmic characters. Moreover, in cultures of young rat mesenchyme, cells similar in general appearance, morphology, and behaviour may be detected round the periphery of the new growth after several hours' cultivation *in vitro*. The evidence in hand at the present moment strongly suggests that these cellular elements are purely stromal in origin.

Bearing in mind our own conclusions, based upon the general behaviour and appearance of these living cells *in vivo* composing the sarcoma cultures, that the pseudo-fibroblasts are merely morphological variations of each other, still more striking are the recent contentions of Carrel and Ebeling (3). These investigators, while working upon a rat sarcoma, succeeded in isolating a pure strain of neoplastic fibroblasts; and they further found that these cells, after cultivation *in vitro* for certain periods, gave rise to tumours after inoculation into healthy, normal rodents. These authors have also detected several differences between the normal fibroblasts and those composing a malignant growth. They discovered that normal fibroblasts are unable to multiply when placed in a medium of blood serum, while on the other hand the cancerous pseudo-fibroblasts are able to proliferate.

A cytological difference was also depicted in the aspect of the general protoplasm between the normal and neoplastic cell.

Further, while critically comparing the appearance of two different strains of sarcomatous fibroblasts with those of the normal type, as seen in both fixed and living cultures, we were able to detect certain structural differences which were found to be specific to each cell-type. Examination of the pseudo-fibroblasts composing the cultures of a 37 C sarcoma reveals them to be spindle-shaped structures (see figures 13, 14), while those of the L.R.S., as has already been demonstrated, possess a typical bifurcated appearance (see figure 12). At first the possibility suggested itself that the morphological differences existing between these two different strains of malignant fibroblast-like elements may in reality be a variation due to the effect of a culture medium upon these cells, and not a true specific one. However, more prolonged study of these cellular components grown in cultures of different media demonstrated quite clearly that a true specific morphological difference exists between these two cell-types. Similarly, when neoplastic fibroblasts are compared morphologically with true normal fibroblasts, a structural variation can again be detected. The normal fibroblasts, on the other hand, are more triangular in general outline, and their nuclei are spherical and small (see figure 11). More detailed observations upon the morphology of these cells cultivated in different media showed that they all conformed to this type.

SUMMARY.

A Lumsden rat sarcoma was cultivated *in vitro*, in a medium composed of chicken embryonic extract, together with either rat or chick plasma. Cultures

grown in a supporting medium consisting of the former type of plasma proliferated much more rapidly than those cultivated in a mixture containing the latter.

The behaviour of the cell-types during growth and differentiation of the cultures was observed, the apparent malignant component was determined, and the general cytology of the explants has been described.

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EXPLANATIONS OF PLATES.

Plates 1 and 2.

Microphotographs depicting unstained living cells composing a Lumsden Rat Sarcoma, taken around the periphery of actively growing cultures after 28 hours' growth in vitro in a medium of plasma and embryonic extract.

Figures 1 and 4. Showing general field around periphery of culture. The cell types are clearly seen, the internal structure of the cells, together with a variable number of highly refractive cytomicrosomes, are clearly depicted. Several cells are seen undergoing pseudopodial activity: in some cases the pseudopodia have burst, liberating the mitochondria and fat globules into the medium.

Figure 2. The same, showing round, tumour cells. The mitochondria and fat droplets show up as highly refractive bodies. To the left of the field several round sarcoma cells may be observed sending out needle-like processes in a radial manner from the body of the cell.

Figure 3. Showing newly-formed pseudo-fibroblast, together with several round tumour cells. The general cytoplasm of the cells is seen crowded with cell granules. The nuclei of these cells are clearly depicted.

Plates 3 and 4.

Microphotographs depicting unstained living cells composing a Lumsden Rat Sarcoma, taken around the periphery of actively growing cultures after 28 hours' growth in vitro in a medium of plasma and embryonic extract.

Figures 5, 7, and 8. Several cells are seen undergoing active pseudopodial movement, the nuclei as well as the internal structure of the cell are clearly seen. Note the arrangement of the highly refractive mitochondria in the pseudopod of Figure 5.

Figure 6. Showing a young active bifurcated pseudo-fibroblast. Many highly refractive mitochondria are shown in the homogeneous protoplasm. Note structure of body in the anterior portion of the cell lying in close relation to the nuclear membrane, which has the appearance of Golgi material.

Plate 5.

Microphotographs of living unstained cells composing cultures of a Lumsden Rat Sarcoma cultivated in plasma and embryonic extract.

Figure 9. Showing photograph of cells in the transitional period taken around the periphery of a healthy culture after 28 hours' growth *in vitro*. Note the refractive cell granules within the clear homogeneous protoplasm.

Figure 10. Photograph depicting the appearance of a four days old culture. Observe the unhealthy appearance of the cells and also the accumulation of numerous large refractive oil droplets.

Plates 6 and 7.

Microphotographs taken in the peripheral region of cultures after 48 hours' growth in vitro: after fixation by osmic vapour and staining with Heidenhain's haematoxylin.

Figure 11. Showing normal healthy fibroblasts. Observe the triangular outline of the cells, together with their small spherical nuclei. Note the deeply stained mitochondria and also the larger faintly stained fat globules. Several large droplets may be seen in the vicinity of the nuclear membranes of the cells.

Figure 12. Depicting two pseudo-fibroblasts of a Lumsden Rat Sarcoma taken in the peripheral region of a 28 hours' culture. Note the characteristic bifurcation of the cells.

Figures 13 and 14. Showing typical spindle-shaped pseudo-fibroblasts composing 48 hours' culture of the 37 C Sarcoma.

Plate 8.

Microphotograph of a clasmatocyte taken around the periphery of a 28 hours' growth in vitro of a Lumsden Rat Sarcoma cultivated in plasma and embryonic extract, after fixation in a 2% Ringer formalin solution and afterwards stained in Heidenhain's iron haematoxylin.

Figure 15. Showing structure of a typical clasmatocyte. Note the extremely irregular outline due to active pseudopodial movement.

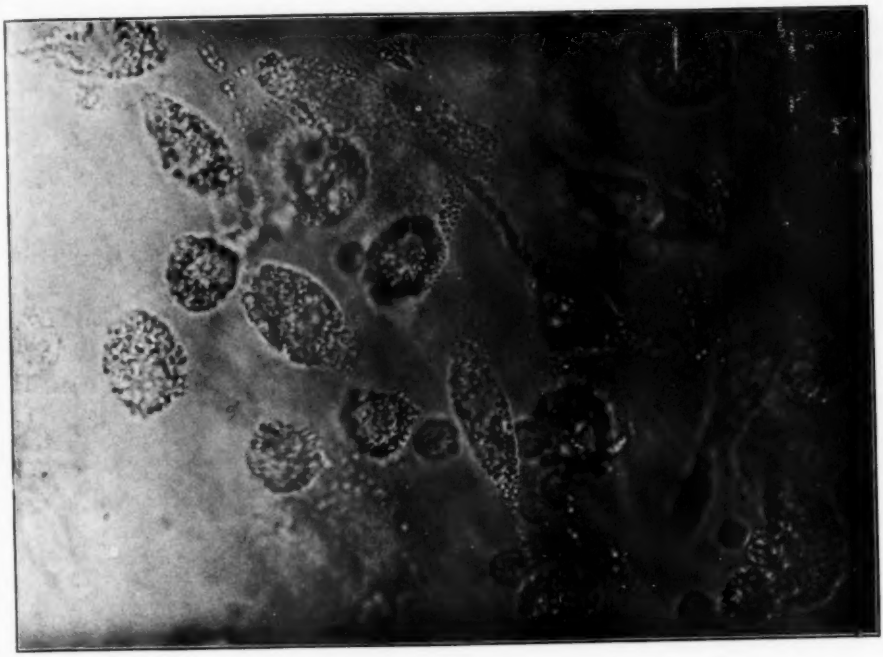

Fig. 1.

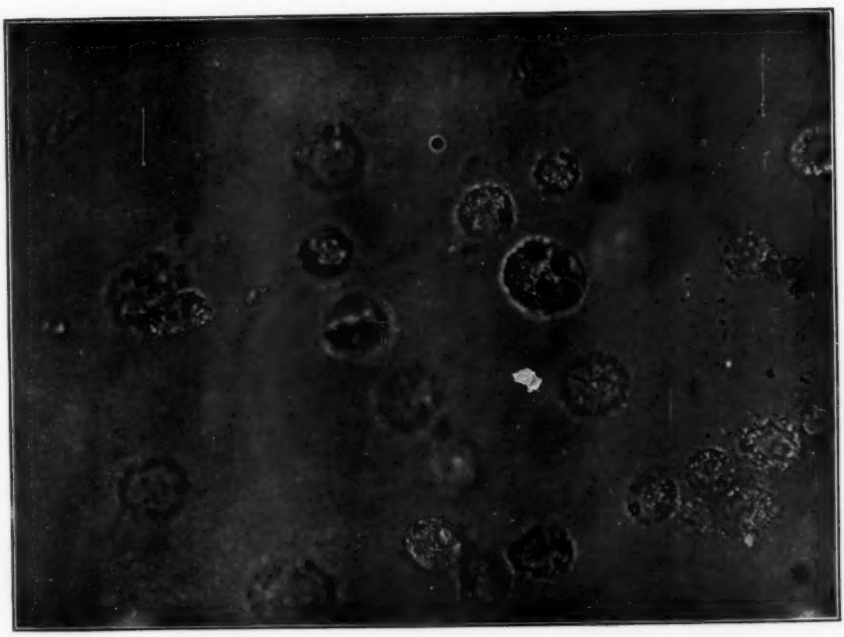

Fig. 2.

PLATE 1.

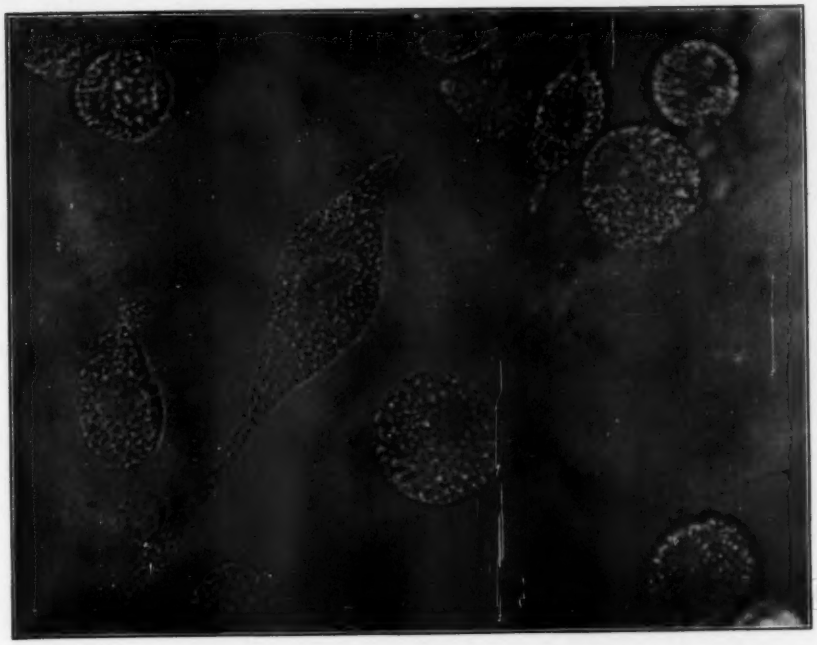

Fig. 3.

Fig. 4.

PLATE 2.

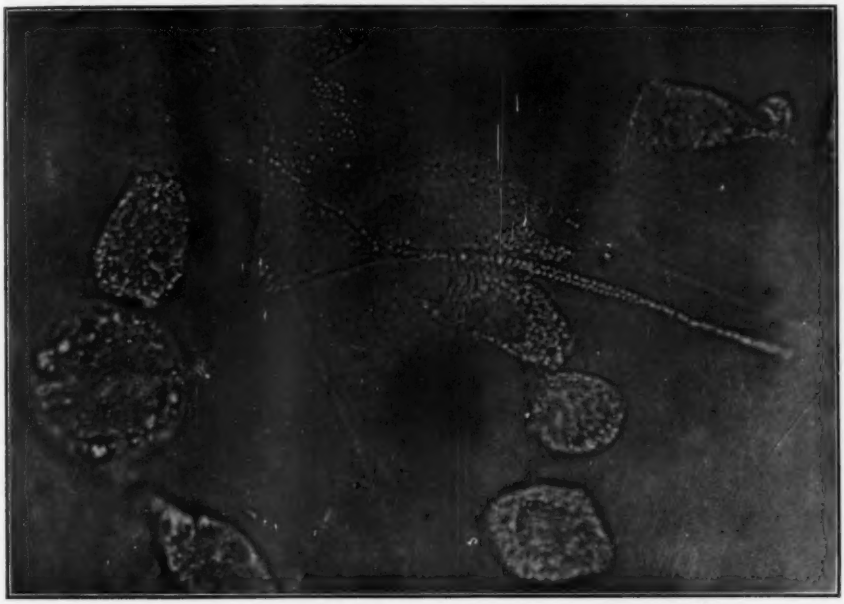

Fig. 5.

Fig. 6.

PLATE 3.

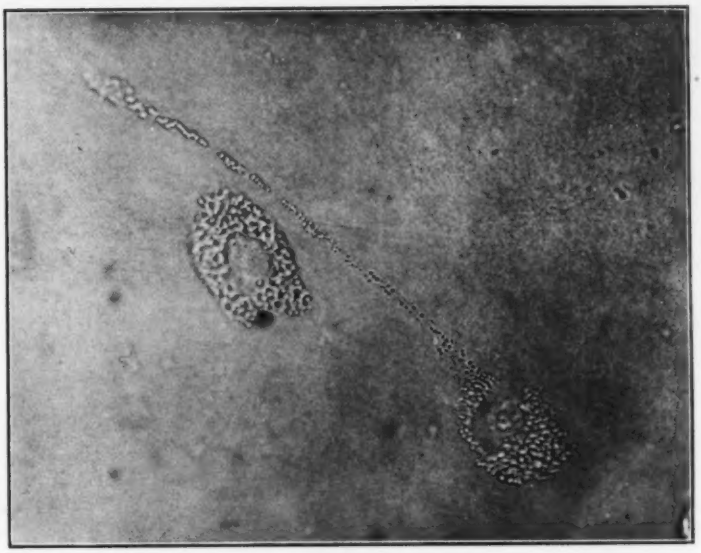

Fig. 7.

Fig. 8.

PLATE 4.

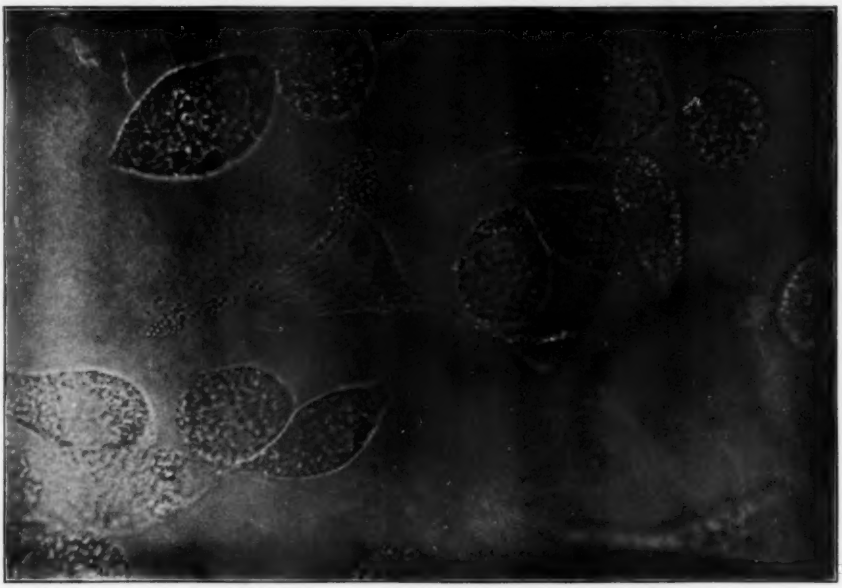

Fig. 9.

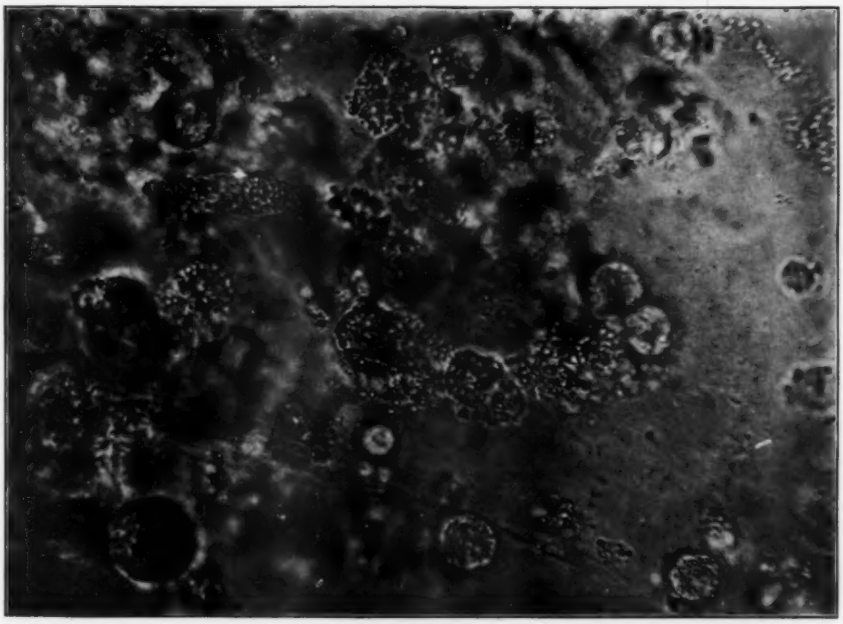

Fig. 10.

PLATE 5.

Fig. 11.

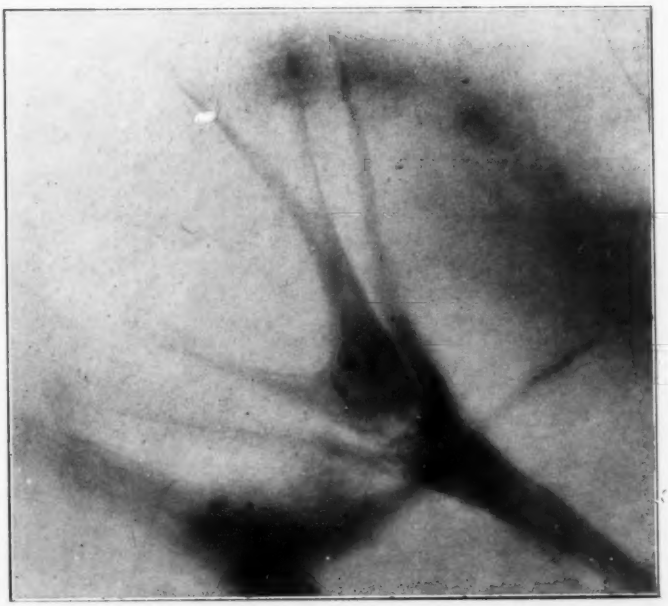

Fig. 12.
PLATE 6.

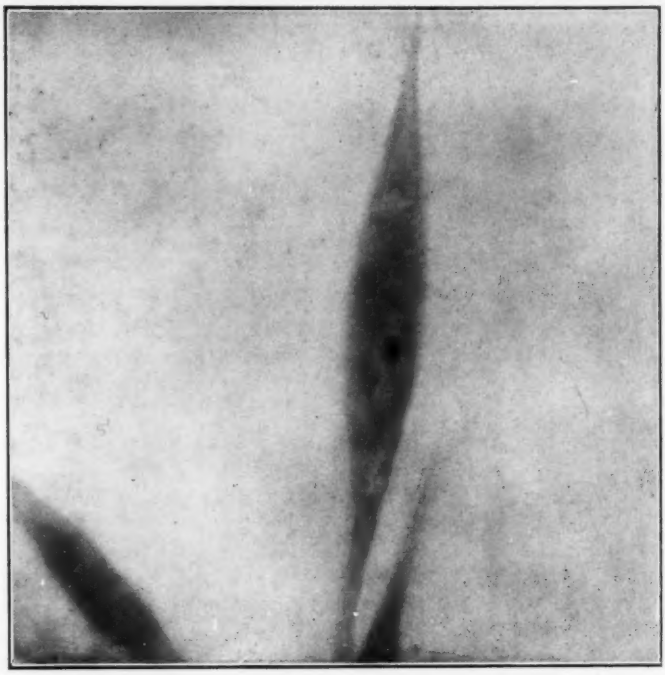

Fig. 13.

Fig. 14.
PLATE 7.

Fig. 15.

PLATE 8.

THE VENOMS OF *OXYURANUS MACLENNANI* AND OF *PSEUDECHIS SCUTELLATUS*[1]

by

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In Northern Queensland there are found two species of very large elapine snakes. One of these is *Oxyuranus maclennani,* described by J. R. Kinghorn (1923) from two specimens obtained by Mr. W. McLennan, who, after killing the snakes, procured their venom by expressing it from the poison glands. These two samples of venom, now some years old, after being powdered and dried *in vacuo* over calcium chloride, weighed 0·475 and 0·356 grammes respectively. They were recently sent to us from the Australian Museum by Mr. Kinghorn. A little earlier, through the kindness of Mr. Donald Thomson, we had become possessed of a third sample of venom, also collected in the field by Mr. McLennan from a large brown snake which he believed to be identical with those of which specimens had been sent to the Australian Museum, and which had been described as *Oxyuranus.* Mr. Kinghorn, in sending the two authentic specimens of *Oxyuranus* venom, warned us that this third sample might prove to be that of another large snake, *Pseudechis scutellatus* Peters, indistinguishable from *Oxyuranus* in its external characters, and differentiated by certain osteological and dental characters. The investigation of this third sample of venom has led us to the belief that this is the case, and we are provisionally describing it as the venom of *Pseudechis scutellatus.*

These two snakes are the largest venomous snakes in Australia. They are from 8 to 10 feet long. *Oxyuranus* has a formidable biting apparatus, and the large venom yield and the high potency of its venom makes it beyond question the most dangerous snake in this country. *Pseudechis scutellatus* must also be regarded as highly dangerous, though its venom is much less potent. We have not attempted to determine finally the toxicity of these venoms, using large numbers of animals, since the samples of *Oxyuranus* have undergone some deterioration, neither being completely soluble in physiological saline, and since the sample obtained from Mr. Thomson is not certainly identified as the venom of *Pseudechis scutellatus.*

1 This research was carried out under a grant from the Department of Health, Commonwealth Government.

1. *The Venom of Oxyuranus maclennani.*

For the estimation of toxicity the larger sample of venom was used. This had been extracted from the venom glands of a snake 8 feet 6 inches long and 7 inches in girth, from which a skull, now in the Australian Museum, was obtained. This sample before being powdered was a hard, translucent mass, which had shrunk from the sides of its containing vessel.

The finely powdered venom, in weighed amounts, was placed in physiological saline, and after an hour at room temperature the insoluble residue was removed by centrifugation before the solution was used for testing. The doses recorded are those of the original dried venom, though only the soluble portion of this has been injected. Since the venom was obtained immediately after death by expression from the venom glands, it may possibly have been diluted to some extent with saliva. For these reasons the venom of this snake must be regarded as even more toxic than our results indicate.

The Results of the Injection of Oxyuranus maclennani in Monkeys.

Three monkeys *(Macacus rhesus)* were injected subcutaneously with varying doses of this venom, in a concentration of 2·0 mgm. per c.cm. The injections were made just below the knee.

Monkey 1. 3·16 kilos, received 1·2 mgm. per kilo. He went off his food, and became weak and ataxic in his movements. Twenty hours after the injection he fell over and lay on his side, without dyspnoea or any distress. The respirations were quiet and entirely diaphragmatic, about 16 per minute. The limbs were not completely paralyzed, occasional movements being observed. There was no circulatory collapse, and the animal remained warm till shortly before death, which took place quietly, without convulsions, 24 hours after the injection. At no stage was any ptosis observed, as with other venoms. The facial muscles contracted rhythmically until shortly before the end, the angles of the mouth being drawn back with each breath. The pupils were neither dilated nor constricted.

Post-mortem. There was no local lesion at the site of injection. The blood was fluid, and the clotting time 14 minutes. There were no gross lesions in any of the organs.

Monkey 2. 2·6 kilos, 1·0 mgm. per kilo injected subcutaneously. After 29 hours the animal was not eating, and there was some weakness and ataxia in the movements. Forty-six hours after the injection the animal fell over, and the only movements observed were those of the facial muscles contracting with each respiration and the passive movements of the abdominal wall. The animal was warm and the heart beating strongly. Under full ether anaesthesia the carotid was cannulized. The mean blood pressure was 90 mm. Hg., the rise and fall at each beat being from 106 mm. to 74 mm. The rate of the heartbeat was 74 per minute, and of the respirations 15 per minute. Stimulation of

the sciatic nerves evoked a good response in the innervated muscles, but there was no appreciable rise in the blood pressure. Stimulation of the phrenic nerves produced a brisk contraction of the diaphragm. The animal died of asphyxia when its thorax was open, and there was no rise in blood pressure. Apart from a few haemorrhages in the lungs there were no post-mortem changes due to the venom.

Monkey 3. 2·71 kilos; 0·5 mgm. per kilo of venom was administered subcutaneously. This animal showed no symptoms, and remained perfectly well.

The lethal dose of this sample of venom for monkeys appears to be about 1·0 mgm. per kilo. The action of the venom is predominantly neurotoxic, and appears to be on the central nervous system, and not on the nerves or nerve-endings, though, as will be seen later, in one or two rabbits the response of the muscles innervated by the sciatic and phrenic nerves was feeble when tested immediately after death while the heart was still beating.

Though the evidence so far as it goes suggests a central rather than a peripheral action of the venom, it should be remembered that Houssay and his colleagues (1922) have produced good evidence that nearly all the venoms they have investigated, including that of *Notechis scutatus*, the Australian tiger snake, have a curarizing action on nerve-endings in skeletal muscle. Whether stimulation of the phrenic will or will not cause brisk contraction of the diaphragm just before death, while the heart is still beating, appears to depend on the time during which the venom has been acting, and upon whether the dose is so large as to produce rapid death from other causes. It seems probable, in the light of Houssay's experiments, that the venom of *Oxyuranus* also has this peripheral action, and experiments to elucidate this point and to decide whether there is a central action as well, are in progress.

The Results of the Injection of the Venom of Oxyuranus maclennani in Rabbits.

Most of the rabbits used were domestic long-eared strains, but a few were young wild rabbits, as indicated by (wild) in brackets after the weight. The results of subcutaneous injection of the venom in a concentration of 2·0 mgm. per c.cm. into the subcutaneous tissues of the abdominal wall are set out in Table 1.

TABLE 1.

The Results of the Subcutaneous Injection of the Venom of Oxyuranus maclennani in Rabbits.

Weight in kilos.	Dose in mgm. per kilogram.	Result.
1·73	2·0	Died in between 6 and 15 hours.
1·84	1·5	Died in between 6 and 15 hours.
2·16	1·0	Died in less than 24 hours.
1·59	0·5	Died in 23½ hours.
1·43	0·3	Paralyzed in 18 hours and remained so till the end of the second day. Survived.

The lethal dose is in the neighbourhood of 0·5 mgm. per kilo. Unfortunately, only one of these animals (that which received 0·5 mgm. per kilo) was observed throughout its illness. After 21 hours it was completely paralyzed, save for the muscles of the face and the muscles of respiration. The respirations were shallow, rapid, and regular. The circulation appeared good, and the animal remained warm till shortly before death. Death took place by failure of respiration. The heart was beating strongly when the thorax was opened immediately before death, and stimulation of the sciatic and phrenic nerves at this time caused only a feeble response in the innervated muscles, though these reacted briskly to direct stimulation. The clotting time was 4½ minutes. Apart from a few petechial haemorrhages at the site of injection, and variable haemorrhages in the lungs and thymus, no gross post-mortem changes were observed in any of these animals.

The results of intravenous injection are set out in Table 2. The venom was injected into a marginal vein of the ear in a concentration of 2·0 mgm. per c.cm., except in the case of the last three animals in the table, in which it was used in a solution containing 0·1 mgm. per c.cm.

TABLE 2.

The Results of the Intravenous Injection of the Venom of Oxyuranus maclennani in Rabbits.

Number of animals.	Average weight in kilos.	Dose in mgm. per kilogram.	Result.
1	1·71	0·9	Died in 5 minutes.
3	2·15	0·8	Died in 3 minutes, 5 minutes, and in 1 hour 39 minutes.
2 (wild)	1·45	0·6	Died in 8½ minutes and in 4 hours 41 minutes.
2	1·83	0·6	Died in 1 hour 20 minutes and in 2 hours 3 minutes.
1	1·89	0·4	Died in less than 24 hours.
1	2·14	0·2	Died in 3 hours.
1	2·0	0·1	Died in 5 hours.
1	2·07	0·05	Severe paralysis on the second day. Survived.
1	1·46	0·03	Paralysis of the hind limbs during the third day. Survived.

The animals which received the smallest doses exhibited striking neurotoxic symptoms, but survived, and those which received 0·8 and 0·9 mgm. per kilo died following intravascular coagulation with typical symptoms (sudden collapse and convulsions). In these there was fibrin whipped out in the right side of the heart, and patchy thrombosis in the portal and mesenteric veins. In no case was massive coagulation observed. Since the "thrombase" factor in venoms is usually more readily destructible, being associated with the coagulable fraction of the venom, it seems likely that the fresh venom, or venom collected and stored under better conditions, would be found to be much more potent in its coagulant action. This suggestion is supported by the examination of the second specimen, which was "denatured" to a greater extent, and was found to have

no demonstrable coagulable action *in vitro*. Injected intravenously into rabbits, in a dose of 2·0 mgm. per kilo, it caused death in 2½ hours, with neurotoxic symptoms, but without any evidence of intravascular clotting. For this reason no attempt has been made to determine accurately the subcutaneous-intravenous index (Fairley, 1929), which is probably greater than 5·0, the certainly lethal dose by the intravenous route being about 0·1 mgm. per kilo.

Animals which, owing to insufficient dosage, escaped the coagulant action of the venom, died quietly without convulsions, with paralysis and failure of respiration. These presented no symptoms immediately following the injection, and, indeed, remained apparently well until a short time before death. The heart was still beating when the thorax was opened after unconsciousness had supervened, the blood was everywhere fluid, and coagulation was somewhat delayed, partial clotting occuring in 7½ to 15 minutes. The phrenic and sciatic nerves were relatively insensitive to faradic stimuli, though the muscles reacted briskly. There were no gross lesions in the organs.

The Effects of the Venom of Oxyuranus maclennani in Guinea-pigs.

The results of subcutaneous injection in the tissues of the abdominal wall of guinea-pigs are shown in Table 3. The venom was used in a concentration of 0·02 mgm. per c.cm.

TABLE 3.

Results of Subcutaneous Injection of the Venom of Oxyuranus maclennani in Guinea-pigs.

Number of animals	Average weight in grams.	Dose in mgm. per 100 grams.	Result.
4	255	0·01	All died in less than 18 hours.
6	215	0·008	Two died in less than 18 hours, one in 24 hours, one on the second day, and two on the fifth day.
2	280	0·005	No symptoms. Survived.

The certainly lethal dose of this sample of venom appears to be about 0·008 mgm. per 100 grammes.

The symptoms following injection are very similar to those observed in rabbits. The animals appear normal till the onset of paralysis, which progresses rapidly, till death occurs from respiratory failure. There is no wetness of the mouth, no obvious dyspnoea, and the circulation appears to be well maintained till shortly before death. Apart from petechial haemorrhages at the site of injection, and haemorrhages in the lungs, there were no obvious pathological changes found at autopsy. The clotting time was normal, 3 to 4 minutes.

The Effect of the Venom of Oxyuranus maclennani in Rats.

A few rats were injected subcutaneously with the venom in a concentration of 0·2 mgm. per c.cm. The results are set out in Table 4.

TABLE 4.

The Results of the Subcutaneous Injection of the Venom of Oxyuranus maclennani in Rats.

Number of animals	Average weight in grams.	Dose in mgm. per 100 grams.	Result.
2	135	0.08	Both died in less than 18 hours.
6	140	0.06	One died in 18 hours, one in 22 hours, one in 28 hours, two on the second day, and one on the fifth day.
2	145	0.04	One died on the fifth day and one survived.
2	150	0.02	Both survived without symptoms.

The lethal dose is about 0·06 mgm. per 100 grammes. The symptoms were closely similar to those observed in guinea-pigs and rabbits, and were predominantly neurotoxic, death occurring from respiratory failure. Apart from haemorrhages in the lungs there were no obvious lesions at autopsy.

The Coagulant Action of the Venom of Oxyuranus maclennani.

This was further investigated, using Lamb's (1903) technique on the citrated plasmas of the rabbit, guinea-pig, and sheep, and on two samples of human plasma. The tests were performed in glass tubes 7·5 cm. long and 12 mm. in internal diameter. These were specially cleaned by boiling in *aqua regia*, and afterwards thoroughly washed in distilled water and dried in the hot-air oven. The venom, in a volume of 0·1 c.cm. of saline, was placed in the bottom of these tubes, and citrated plasma (prepared by shedding blood into an equal volume of 2% sodium citrate in physiological saline and centrifuging out the corpuscles) was added in a volume of 0·2 c.cm., using a dropping pipette. The clotting times in the tubes were observed minute by minute at 37°C. The results are set out in Table 5, where for comparison the clotting times of one of the human plasmas with black snake venom are shown.

TABLE 5.

The Coagulation of Plasma with Oxyuranus Venom in vitro.

Amount of venom in mgms.	Clotting times in minutes.						
	Guinea-pig. (1)	Guinea-pig. (2)	Rabbit.	Sheep.	Human. (1)	Human. (2)	Black Snake. Human. (2)
0.1	15	15	1½	30	5	9	8
0.05	25	20	2	60	7	10	8
0.25	40	40	3		13	12	10
0.012	90	90	5		22	20	18
	(soft)			not			
0.0062	not		10	clotted	30	60 (soft)	25
0.0031	clotted		20	in			
0.0016	in		30	20 hours	not clotted in 20 hours		
0.00078	20 hour		20 hours				

The differences between plasmas from different species may be dependent on genuine differences in the reaction of the species which with other venoms

have been demonstrated by intravenous injection, or they may be in part related to the methods of bleeding. The human blood was obtained from the median basilic vein, rabbit blood from the marginal vein of the warmed ear, and guinea-pig and sheep blood from the divided vessels of the neck, the citrated sheep blood being procured at the abattoirs.

Experiments with the second smaller sample of venom, to which it was suspected spirit had at some time been added, were of some interest because, though we failed to demonstrate any trace of coagulant activity *in vitro* with any of these plasmas, even using 2·0 mgm. of venom to 0·2 c.cm. of plasma, the venom exhibited a feeble anticoagulant action in doses of 0·1, 0·05, 0·025, 0·012, and 0·0062 mgm. For example, after incubation with guinea-pig plasma for 1 hour, the addition of calcium chloride 0·5% solution in an amount sufficient to cause clotting in control tubes within 4 minutes, only resulted in clotting after the lapse of 50 minutes with 0·1, 0·05, and 0·025, of 40 minutes with 0·012, and of 15 minutes with 0·0062 mgm. Plasma incubated with smaller amounts of the venom exhibited normal clotting on the addition of calcium chloride.

The Haemolytic Effects of the Venom of Oxyuranus maclennani.

The haemolytic action of the venom was tested on the red blood cells of man and of the sheep, rabbit, and guinea-pig. The cells were used in a 3% suspension. The total volume of the haemolytic system was 0·3 c.cm. (0·1 c.cm. of saline or added guinea-pig serum, 0·1 c.cm. of saline containing venom, and 0·1 c.cm. of 3% suspension of red blood corpuscles). "Washed" red blood cells were three times washed in normal saline by centrifugation, and "unwashed" cells were made up at once after spinning them down from the citrated blood. A few experiments were also made using defibrinated blood diluted with saline so as to contain the same proportion of red blood corpuscles as the 3% suspensions in saline.

The tubes used for these haemolysis tests were boiled in *aqua regia*, thoroughly washed in distilled water, and subsequently dried in the hot-air oven. The venom was used in doses of 0·1 mgm., $0·1 \times 10^{-1}$ mgm. . . . . $0·1 \times 10^{-9}$ mgm., or in a closer spaced series, 0·1, 0·05, 0·025, 0·012 . . . . 0·000195 mgm.

With *human* red blood cells, both "washed" and "unwashed," and venom in doses of 0·1, $0·1 \times 10^{-1}$ . . . . $0·1 \times 10^{-9}$ mgm., there was no haemolysis after 4½ hours at 37°C. The addition of 0·1 c.cm. of guinea-pig serum to either "washed" or "unwashed" cells after incubation with venom for 3 hours caused traces of haemolysis in the first three tubes in the series after a further 45 minutes' incubation. Red blood cells from another individual were tested with 0·1, 0·05 . . . . 0·000195 mgm. of venom. Incomplete haemolysis of "washed" cells was observed with 0·1 mgm. of venom after 2½ hours, and complete haemolysis with 0·1 and 0·05 mgm. of venom after 3 hours, but no haemolysis was observed in the parallel experiment with defibrinated blood. In the case

of "washed" cells, incubation in the presence of complement caused some haemolysis in all the tubes in 15 minutes and complete haemolysis in ¾-hour, but with defibrinated blood no haemolysis was observed until ¾-hour, and then only in the first four tubes, containing 0·1, 0·05, 0·025, and 0·0125 mgm. of venom. After 1¼ hours the first seven of the ten tubes in the series were completely haemolyzed. This experiment affords an excellent example of the "protective" action of homologous serum, like that described by Duhig and Jones (1928) in their experiments on the venom of the stone fish, *Synanceja horrida*. A similar phenomenon was noted by Welsh and Chapman (1910) in their study of haemolysis by Australian venoms.

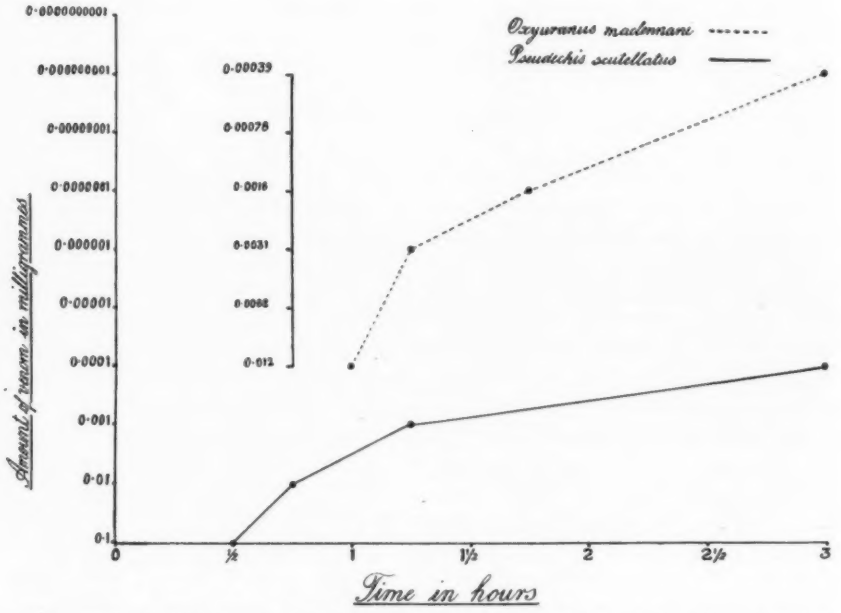

Figure 1. Graphic representation of haemolysis of washed cells of the guinea-pig by the venom of *Oxyuranus maclennani* is doses of 0·1, 0·05, 0·025, 0·012 . . . . 0·00039 mgm., and of the venom of *Pseudechis scutellatus* in doses of 0·1, 0·01, 0·001, and 0·0001 mgm.

With *sheep* red blood cells no haemolysis was observed in 20 hours, and the addition of complement failed to cause any haemolysis. With *rabbit* red blood cells both "washed" and "unwashed," there was also no haemolysis, but the effect of the addition of guinea-pig serum was not tested.

Guinea-pig red blood cells (figure 1) were much more sensitive to this venom. Complete haemolysis of "unwashed" cells took place with 0·1, 0·05,

0·025, and 0·012 mgm. in 1 hour, with 0·031 mgm. of venom in 1 hour 20 minutes, with 0·0016 in 1¾ hours, and with 0·00078 and 0·00039 mgm. in 3 hours.

The experiments with human, rabbit, and guinea-pig red cells demonstrate the feeble nature of the haemolytic action of this venom, a result which was already suggested by the post-mortem findings in animals killed with venom.

Experiments with guinea-pig red cells showed only a very slight difference in haemolytic activity between the two samples of this venom. The haemolytic, unlike the coagulant, activity of the venom, has not been greatly impaired by the excessive denaturation which has occurred in the smaller sample.

The Effects of the Venom of Oxyuranus on the Circulation.

A number of experiments of a preliminary nature have been made on cats and rabbits under ether anaesthesia without artificial respiration, in which the blood pressure has been recorded by cannulization of the carotid artery. In some the vagi have been intact, and in others divided. The venom used in most of these experiments has been the smaller ("denatured") sample, which does not cause intravascular coagulation when injected intravenously.

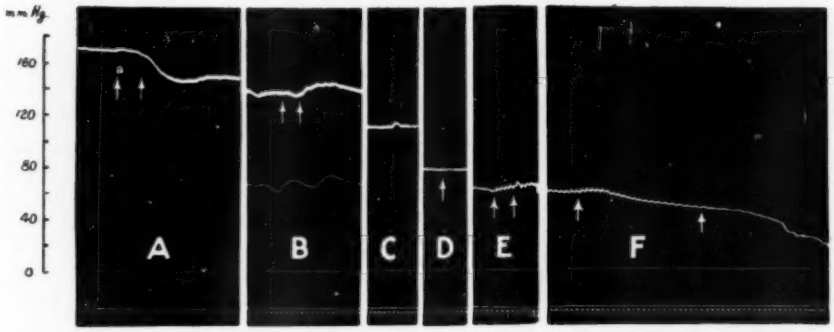

Figure 2. Blood pressure tracing from a cat (Protocol 1) which was injected at A with 10 mgm. of *Oxyuranus* venom intravenously. At B a second dose of 8·0 mgm. was administered. A portion of a plethysmograph record of the small bowel is shown here. As this appeared to be recording only passively the bowel was restored to the abdomen. C, tracing at 1.46 p.m. (Protocol 1). D, record at 1.53 p.m.; vagi cut, no rise of pressure resulting. E, record at 2.20. Right sciatic nerve stimulated, only a very small rise of pressure. F, 5·0 mgm. of *Oxyuranus* (undenatured); rapid fall of blood pressure with coagulation in the right heart.

It has been administered intravenously in a concentration of 2·0 mgm. per c.cm., the injections being made slowly from a syringe. Rabbits were found to be more suitable than cats, as they are much less resistant to the action of the venom, and a dose of about 10 mgm. is sufficiently large to produce death within an hour or two, obviating the necessity for prolonged anaesthesia.

It was hoped that by first injecting a dose of denatured venom it might be possible to induce a negative phase, as Martin (1894) did by injection of minute doses of coagulant venoms, and so permit of the study of the less denatured sample of venom used in most of the other experiments, by injection at a sufficiently rapid rate to ascertain its immediate effects on the blood pressure. This was not possible, and the injection of this sample of venom invariably caused a rapid fall of blood pressure associated with intravascular clotting. Protocols 1 and 2 illustrate the general effects of the venom in the cat and rabbit.

The immediate effect of the injection of a large dose of this venom is to cause a small fall in blood pressure. This is illustrated in figure 2 at A, where a dose of 10 mgm. of *Oxyuranus* was injected. The pressure fell from 169 mm. to 145 mm., shortly recovering to 148 mm. A condensed protocol of this experiment is given here.

Protocol 1. Cat, 2·24 kilos.

12.10 p.m. Ether anaesthesia. Trachea and carotid cannulized. Vagi intact. Time marker, 5 second intervals.
12.34 p.m. Blood pressure recording, 180 mm. Hg.
12.54 p.m. 10 mgm. Oxyuranus (''denatured'' sample) injected into femoral vein. Blood pressure 169, falls to 145 mm. and rises again to 148 mm.
1.12 p.m. Blood pressure 142 mm.
1.23 p.m. Blood pressure 135 mm. 8·0 mgm. Oxyuranus ''denatured'' venom injected.
1.46 p.m. Blood pressure 110 mm.
1.53 p.m. Blood pressure 76 mm. Vagi cut, no rise in blood pressure.
2.20 p.m. Blood pressure 62 mm. Right sciatic nerve stimulated, very slight rise in blood pressure to 66 mm. Good contraction of innervated muscles.
2.25 p.m. Blood pressure 60 mm. 5·0 mgm. of undenatured Oxyuranus venom. Rapid fall in blood pressure and death in four minutes with fibrin whipped out in the right heart but no other sign of intravascular clotting.

Protocol 2. Rabbit, 2·6 kilos. Ether anaesthesia; trachea and carotid cannulized, Vagi cut.

3.50 p.m. Blood pressure recording. 135 mm. Hg.
3.52 p.m. Blood pressure 130 mm. 8·0 mgm. denatured Oxyuranus venom. Blood pressure falls to 117 mm.
3.57 p.m. Respirations 88 per minute. Blood pressure 123 mm.
4.10 p.m. Blood pressure 120 mm. 1·0 mgm. Oxyuranus venom (undenatured). No change in blood pressure.
4.14 p.m. Respirations 80 per minute.
4.16 p.m. Blood pressure 122 mm. 1·2 mgm. Oxyuranus venom (undenatured).
4.26 p.m. Blood pressure 125 mm. Traube waves. Blood not so well oxygenated.
4.30 p.m. Blood pressure 126. Traube waves about seven per minute. 1·8 mgm. Oxyuranus venom (undenatured).
Death in four minutes from the commencement of this injection. Traube waves beautifully marked as the pressure falls. Fibrin whipped out in the heart. Stimulation of the phrenic nerves causes brisk contraction of the diaphragm.

Smaller doses of venom (2·0 mgm.) also cause a small immediate fall in pressure, sometimes preceded by a still smaller rise, which is possibly a volume effect. The later effects of a single dose of the venom on the circulation, when the effects of changes in lung ventilation are eliminated by section of the vagi, are illustrated in Protocol 3.

Protocol 3. Rabbit, 2·67 kilos. Ether anaesthesia; trachea and carotid cannulized, vagi divided, and 9 mgm. of *Oxyuranus* administered intravenously.

Time after injection.	Blood pressure.	Respiration Rate.
1 minute	Falls from 64 to 56 mm.	84
6 minutes	56 mm.	87
13 „	74 mm.	
18 „	66 mm.	94
23 „	66 mm.	84
34 „	72 mm.	74
41 „	84 mm. (Feeble output per beat, oxygenation not so good)	48
48 „	78 mm. (Oxygenation improved)	54
55 „	84 mm. (Traube curves)	66
64 „	80 mm.	44
70 „	70 mm.	34
74 „	Blood pressure falls rapidly and respiration ceases. Artificial respiration ineffective.	

The blood pressure for some time is moderately well maintained, though after about an hour the output of the heart per beat becomes very small. At this stage the respiratory rate, which has been steadily declining, becomes slower, and the blood in the arterial cannula is obviously less well aerated. The blood pressure often rises somewhat, possibly owing to asphyxia, and at this stage Traube curves may appear in the tracing. Finally, the respiratory rate becoming progressively slower, there is a sudden fall of blood pressure, respiration failing at about the same time. There are never convulsions, though stimulation of the sciatic and phrenic nerves always causes brisk responses in the innervated muscles. The failure to demonstrate any peripheral action of the venom in these experiments is probably related to the size of the dose employed and the short period of time during which it has been acting.

Further experiments are in progress for the analysis of the action of this and other venoms on the circulation and respiration, using decerebrate preparations to eliminate the complicating effect of anaesthesia, and decapitate and spinal preparations for the study of the effects on the cardio-vascular system under artificial ventilation. The results of these experiments will form the subject of a further communication.

2. *The Venom of Pseudechis scutellatus.*

This sample of venom was in excellent condition, and was completely soluble in water and saline. Its toxic action will be seen to be strikingly different from that of *Oxyuranus maclennani.* Instead of being coagulant, powerfully neurotoxic and feebly haemolytic in its action, it is anticoagulant, not very strongly neurotoxic, but powerfully haemolytic, and resembles rather closely the venom of *Pseudechis guttatus* De Vis, recently examined by one of us (Kellaway, 1929).

The Effect of the Venom on Rabbits.

Four rabbits were injected with this venom, in a concentration of 2·0 mgm. per c.cm., into the subcutaneous tissues of the abdominal wall. One weighing 1·38 kilos received 3·0 mgm. per kilo, and died in 5¾ hours. Another, also a wild rabbit, weighing 1·3 kilos, received 2·0 mgm. per kilo, and died in less than 18 hours. A domestic rabbit, weighing 1·73 kilos, which received the same dose per kilo, and another weighing nearly 2 kilos, which received half this dose, survived without symptoms. The symptoms which were exhibited by the two rabbits which died were similar to those (shortly to be described) exhibited by rabbits injected intravenously, but were longer delayed in onset. The animals appeared to be sick, but no neurotoxic symptoms were observed, haemolytic changes dominating the picture.

Post-mortem.

The abdominal subcutaneous tissues were the seat of an extensive haemorrhagic oedema spreading from the place of injection. The suprarenals were congested, and there was haemorrhagic congestion of the bowel. There was intense haemoglobinuria. The lungs and thymus contained haemorrhages. The blood was fluid in the heart, and great vessels, and its clotting time was delayed. The serum which exuded from the clot was deeply stained with haemoglobin.

The results of intravenous injection were more dramatic. Even with very large doses there were no immediate symptoms suggestive of intravascular coagulation, though death took place very rapidly in one animal. The results are set out in Table 6. The venom was injected in a concentration of 2 mgm. per c.cm., except in the first animal, where it was used in a concentration of 5 mgm. per c.cm.

TABLE 6.

The Results of Intravenous Injection in Rabbits.

Number of animals.	Average weight in kilos.	Dose in mgm. per kilo.	Result.
1	2·02	5·0	Died in 20 minutes.
1	1·54	2·0	Died in 3¼ minutes.
1	1·4	1·0	Died in between 1 hour 20 minutes and 2 hours 50 minutes.
2 (wild)	1·3	0·9	Both died in 1 hour 15 minutes.
2 (wild)	1·25	0·7	Died in 41 minutes and in 1 hour 39 minutes.
2	1·75	0·6	No symptoms. Survived.

The certainly lethal dose is more than 2·0 mgm. per kilo by subcutaneous injection, and about 0·7 mgm. per kilo intravenously. The animal which received the largest dose had no symptoms for 10 minutes. It then exhibited slight weakness, and inco-ordination of its movements. After 19 minutes it had a very brief convulsion, and respiration ceased in 20 minutes. The blood

was everywhere fluid, and the clotting time 10 minutes. The serum which exuded from the clot was deeply blood-stained. There was a little staining of the peritoneum. There were no other lesions. The phrenic and sciatic nerves were stimulated immediately after death, and brisk contraction of the innervated muscles resulted. The symptoms in the remaining animals were very similar. There was a latent interval of varying length, in which there were no obvious symptoms, and then quite suddenly the animal went over on its side and died in a brief convulsive seizure. One animal had a terminal pulmonary oedema, with blood-stained frothy fluid oozing from the mouth and nose. All exhibited intense intravascular haemolysis. Clotting was much delayed, frequently over an hour, and the serum was deeply coloured with blood pigment. The peritoneum and subperitoneal tissues were stained with blood pigment. There was much blood-stained fluid free in the peritoneal cavity, and some animals had similar effusions in the pleural cavities. The bladder in rabbits surviving sufficiently long contained much blood pigment in the urine, and the cut surface of the kidneys was black with altered blood. There was intense haemorrhagic congestion of the small bowel, and there was no obvious clotting anywhere in the vascular system.

Effects in the Guinea-pig.

The effect of subcutaneous injection was also observed in guinea-pigs, and the results of injection in a concentration of 2·0 mgm. per c.cm. are set out in Table 7.

TABLE 7.

Results of Subcutaneous Injection in Guinea-pigs.

Number of animals	Average weight in grams.	Dose in mgm. per 100 grams.	Result.
6	262	0·2	Three died in between 9 and 12 hours, one in less than 18 hours, one in 27 hours, and one survived.
6	261	0·15	Two died on the first day, three on the second day, and one on the third day.
6	268	0·1	One died in less than 18 hours, two on the second day, and three survived.
5	256	0·08	One died on the third day and four survived.

The certainly lethal dose is somewhat more than 0·2 mgm. per 100 grammes. The symptoms were very similar to those observed in rabbits. Apart from a slight roughness of the coat and anorexia, there were no symptoms till shortly before death. The animals did not appear to be paralyzed, though there were sometimes muscular weakness and ataxia. Haemoglobinuria was frequent, and death evidently resulted from the haemolytic action of the venom. The post-mortem findings were closely similar to those found following the injection of diphtheria toxin or of the venom of *Pseudechis guttatus*. All the animals had intense haemorrhagic oedema of the abdominal wall, almost black haemorrhagic

adrenals, and haemorrhagic small bowel. There was much free blood-stained fluid in the peritoneum, and sometimes in the pleural cavities. Haemorrhages in the lungs were constantly present.

The Effects of the Venom of Pseudechis scutellatus in Rats.

The effects of subcutaneous injection of this venom in a few rats are set out in Table 8. The venom was given in the flank in a concentration of 2·0 mgm. per c.cm.

TABLE 8.

The Effects of Subcutaneous Injection in Rats.

Number of animals	Average weight in grams.	Dose in mgm. per 100 grams.	Result.
2	120	0·8	Died in 3½ and 3¾ hours.
6	156	0·4	One died in 23 hours, one in 24 hours, one in 26 hours, two later on in the second day, and one survived.
4	134	0·3	One died in 26 hours, one later on in the second day, and two survived.
2	150	0·2	No definite symptoms. Survived.
2	135	0·15	One died on the third day and the other survived.

The certainly lethal dose is more than 0·4 mgm. per 100 grammes. The symptoms exhibited after injection were very similar to those observed in rabbits and guinea-pigs. The coat was ruffled, and there was often haemoglobinuria. The animals did not become paralyzed, but some appeared to be weak and ataxic before they fell over very shortly before death. The post-mortem findings were similar to those observed in guinea-pigs. There were haemorrhagic oedema spreading from the region of the injection, haemorrhages in the lungs, and haemorrhage and congestion of the bowel. Haemorrhagic adrenals were seldom observed.

The Anticoagulant Action of the Venom.

In vitro experiments were made with this venom side by side with those earlier described on the venom of *Oxyuranus maclennani*. With none of the plasmas was any coagulant action observed with doses of venom up to 2·0 mgm., but, on the contrary, after incubation for 1 hour, and the addition of sufficient calcium chloride to cause rapid coagulation in control tubes, clotting failed to occur in the tubes containing larger amounts of venom in the series 0·1, $0{\cdot}1 \times 10^{-1}$ . . . . $0{\cdot}1 \times 10^{-7}$ mgm. The clotting times after the addition of varying amounts of calcium chloride to the different plasmas which had been incubated with venom, and the clotting times in control tubes, are given in Table 9.

TABLE 9.

Anticoagulant Action of the Venom of Pseudechis scutellatus.

Dose of venom in mgm.	Clotting times in minutes after addition of $CaCl_2$. Guineapig.	Rabbit.	Sheep.	Human.
Nil	4	9½	4	18
.1	No clotting	No clotting	No clotting	No clotting
.01	„	„	„	„
.001	„	„	25 (soft)	„
.0001	12 hours	„	7	„
.00001	4½ mins.	30 mins. (soft)	4½	30 mins.
.000001	4 „	15 „ „	4	25 „
.0000001	4 „	9½ „ „	4	18 „

The anticoagulant action of this venom is much more striking than that of the venom of the copper-head and death adder (Kellaway, 1929), and approximates closely to that of cobra venom.

The Haemolytic Action of the Venom of Pseudechis scutellatus.

The results of the injection of this venom into animals, and particularly those of intravenous injection in rabbits, demonstrate its striking haemolytic activity. Tests *in vitro* with washed human and rabbit red blood corpuscles, using the methods earlier described, in which comparison was made with the

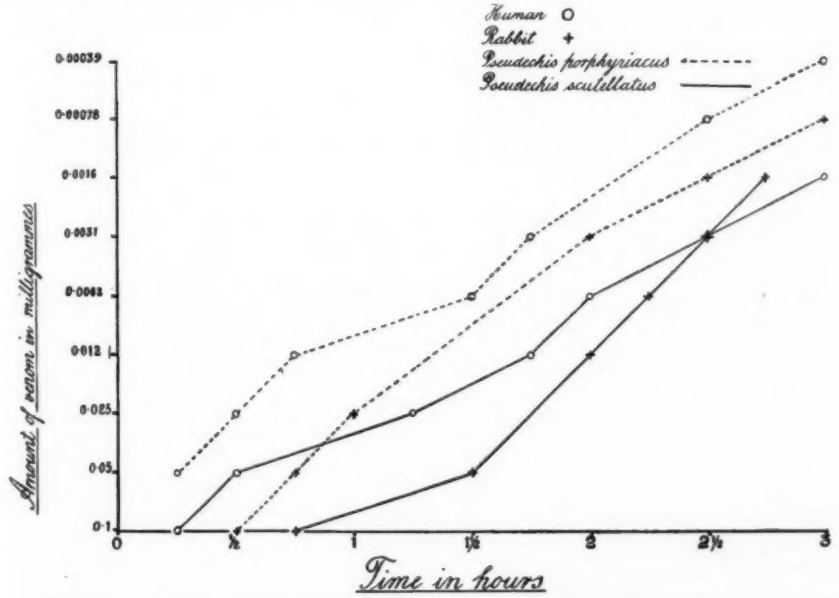

Figure 3. Graphic representation of haemolysis of washed human and rabbit red blood cells by the venom of *Pseudechis porphyriacus* and *Pseudechis scutellatus*.

venom of the black snake *(Pseudechis porphyriacus)*, are graphically represented in figure 3. This is a convenient method of displaying the results, and has no strict quantitative significance. The times at which the various quantities of venom in the series, 0·1, 0·05 . . . . 0·00039 mgm., cause complete haemolysis, furnish the points on the curve. It will be seen that for red blood corpuscles of these two species the venom is but little inferior in activity to that of *Pseudechis porphyriacus*. With defibrinated human blood no haemolysis took place in 3 hours with either venom, but in defibrinated rabbit blood the red cells were hardly protected at all by the presence of homologous serum. The addition of guinea-pig serum to washed human cells and venom caused complete haemolysis in all the tubes in ¾-hour at 37°C., and even with defibrinated blood, though the process was slower, haemolysis was complete in 1¼ hours. With "washed" guinea-pig red blood cells (figure 1) 0·1 mgm. caused complete haemolysis in ½-hour, 0·01 mgm. in ¾-hour, 0·001 in 1 hour 20 minutes. 0·0001 in 3 hours. The sensitiveness of these cells to haemolysis by this venom is therefore about the same as those of man and of the rabbit. With sheep red cells no haemolysis was observed.

The Effect of the Venom of Pseudechis scutellatus on the Circulation.

A number of experiments were made on rabbits under ether anaesthesia without artificial ventilation, in which the trachea and carotid were cannulized and the blood pressure recorded. In some the vagi were intact, and in others they were divided.

The effects of this venom on the circulation were much more striking than those produced by the injection of the denatured specimen of the venom of *Oxyuranus*. The immediate rise and fall of blood pressure produced by doses of 5–8 mgm. intravenously is strikingly like the effect produced by a very small dose of adrenaline, and it is possible that the immediate effect of the venom is actually produced by the liberation of adrenaline from the suprarenals. No proof of this suggestion is, however, offered in the present communication.

Figures 4 and 5 illustrate the circulatory changes which follow the injection of this venom, and the experiments from which they were taken are set out briefly in Protocols 5 and 6.

Protocol 5 (figure 4). Rabbit, 1·44 kilos. Ether anaesthesia; trachea and carotid cannulized, vagi intact. Time in 5-second intervals.

2.50 p.m. Blood pressure 100 mm. Hg.
2.51 p.m. 8·0 mgm. *Pseudechis scutellatus* venom injected intravenously. Blood pressure rises from 91 mm. to 98 mm. and then falls to 55 mm. within 15 seconds.
2.53 p.m. Blood pressure 34 mm.
3.1 p.m. Blood pressure 48 mm. Blood well aerated.
3.5 p.m. Respirations 120 per minute, regular. Blood pressure 60 mm.
3.12 p.m. Respirations 106. Blood pressure 90 mm. Marked Traube curves, 11 per minute.
3.15 p.m. Respirations 96 per minute. Blood not so well oxygenated. Blood pressure 95 mm.
3.16 p.m. to 3.20 p.m. Rapid fall of blood pressure from 80 mm. to 18 mm. Heart fails before respirations which at the beginning of this period are 58 per minute.

Protocol 6 (figure 5). Rabbit, 2·5 kilos. Ether anaesthesia; trachea and carrotid cannulized; loop of bowel enclosed in plethysmograph, and vagi divided. Time in 5-second intervals.

11.50 a.m. 8·0 mgm. *Pseudechis scutellatus* venom injected intravenously. Blood pressure rises from 76 to 85 mm. and then falls in the next 20 seconds to 56 mm. Plethysmograph shows a small increase in volume coincident with this fall. (Plethysmograph record a little behind the blood pressure.)
11.52 a.m. Blood pressure 70 mm.
11.57 a.m. Blood pressure 73 mm. Blood not so well oxygenated.
11.58 a.m. Blood pressure 80 mm. Bowel volume decreases as blood pressure rises.
11.59 a.m. Mean blood pressure 84 mm.
12.6 p.m. Fall in blood pressure.
12.7 p.m. Respiration fails.
12.8½ p.m. Animal dead. The bowel volume falls passively with the blood pressure.

A number of other experiments, both with and without a bowel plethysmograph, was made, and gave closely similar results. In all cases the volume of the bowel increased with the initial fall of blood pressure, and diminished as the pressure rose later. This later rise is probably asphyxial in origin, though the blood in the arterial cannula was not always obviously poorly aerated till later, when the blood pressure was falling. In most of the experiments Traube curves were present in the tracing when the blood pressure was high, just before the dramatic fall of blood pressure which appeared to precede failure of respiration. We have not been fortunate enough to get a good plethysmograph record during the period in which the Traube curves were characteristically present (figure 4 C).

During the final fall the bowel volume was diminished, the plethysmograph record following the blood pressure passively. Protocol 7 is a very good example of the high level of blood pressure reached before the final rapid fall. The plethysmograph record showed vasoconstriction with the rising pressure, and only followed the blood pressure passively in the last 3 or 4 minutes of the experiment.

Protocol 7. Rabbit, 2·70 kilos. Ether anaesthesia; carotid and trachea cannulized; loop of bowel enclosed in a plethysmograph.

4.3 p.m. Blood pressure 66 mm. Hg. Vagi divided. Blood pressures rises to 89 mm. Hg.
4.13 p.m. Blood pressure 90 mm. 5·0 mgm. venom injected. Blood pressure rises to 95 and falls quickly to 80 mm. Hg.
4.16 p.m. Respirations 72 per minute forcible. Blood pressure 93 mm. Hg.
4.17 p.m. Blood pressure 91 mm. Hg.
4.24 p.m. Blood pressure 102 mm. Hg.
4.27 p.m. Blood pressure 106, still rising. Respirations 67 per minute.
4.30 p.m. Blood pressure 120 mm. Respirations 68. Blood still bright in arterial cannula.
4.37 p.m. Blood pressure 140 mm., rising to 150 mm. Hg.
4.42 p.m. Blood pressure 140 mm., falling.
4.43 p.m. Blood pressure 90 mm., falling abruptly to 8 mm.
4.45 p.m. Respiration ceased.

In the last stages of several of these experiments the sciatic and phrenic nerves were stimulated with a faradic current, and brisk contraction was obtained in the innervated muscles.

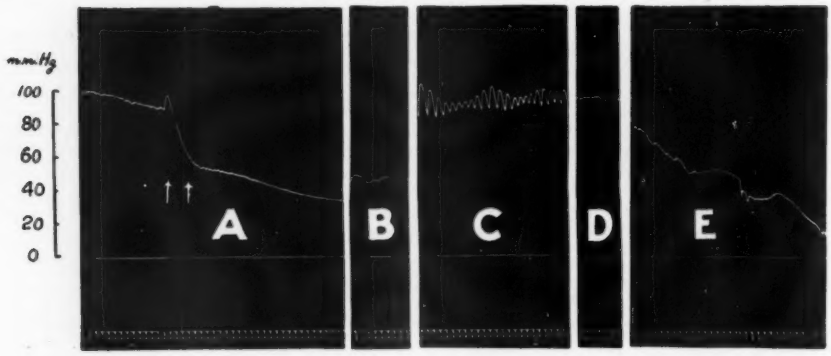

Figure 4. Blood pressure record of a rabbit which received 8.0 mgms. of the venom of *Pseudechis scutellatus* intravenously. At A, injection of venom. B, tracing at 3.1 p.m. (see Protocol 5). C, tracing at 3.12 p.m., showing Traube curves. D, tracing at 3.15 p.m. E, fall of blood pressure, showing effect of terminal convulsive movements.

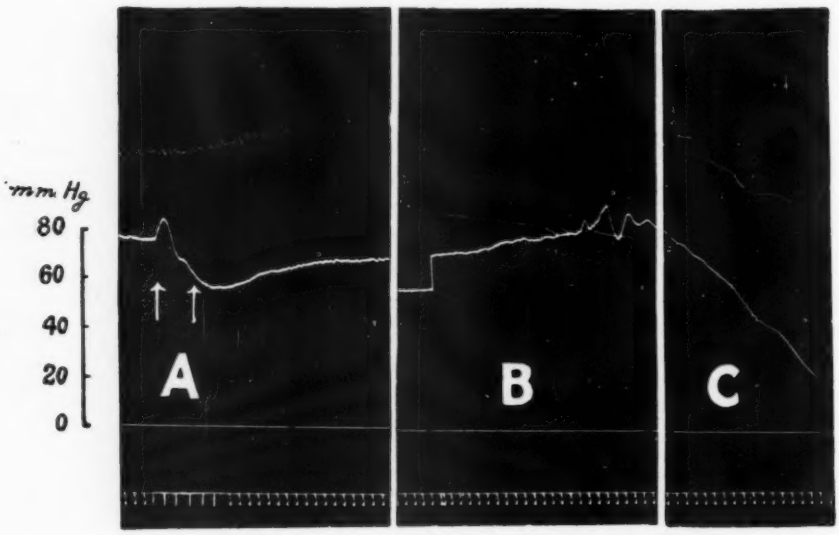

Figure 5. Blood pressure and plethysmograph records from a rabbit following the intravenous injection of the venom of *Pseudechis scutellatus*. The upper tracing is from the bowel plethysmograph. At A, 8.0 mgm. of venom injected. B shows part of the tracing at 12.53 and 12.57 p.m. (Protocol 6). The plethysmograph tracing point is placed a little behind that of the manometer. C shows the final fall of blood pressure; the plethysmograph writing point has been raised by blowing air into the system so that it records above the blood pressure tracing after crossing it in B.

Convulsive movements were not infrequent as a terminal event, and in figure 4 they produce the irregularities in the final fall of blood pressure. We are inclined to the belief that the asphyxial picture produced by this venom is due to intravascular haemolysis interfering with the oxygenation of the bulbar centres, since the animal goes on breathing vigorously, the ventilation of the lungs appearing to be excessive, and the blood in the arterial cannula remaining bright in colour. Respiration ceases when the blood pressure has already fallen to a very low level.

The effects of the venom upon the circulation in this dosage are probably wholly indirect, its first action being the sudden liberation of adrenaline, and its later effect being due to anoxaemia. There is no evidence of neurotoxic action in these rapid deaths, which appear to be due to the haemolytic action of the venom. These conclusions must be regarded as provisional only. Further experiments are in progress for the analysis of the action of this and other Australian venoms on the circulation.

The Stimulant Action of the Venoms on Plain Muscle.

Like all the other Australian elapine venoms so far tested, the two venoms discussed in the present paper have the stimulant action on plain muscle recently described by one of us (Kellaway).[2] On the plain muscle of the guinea-pig the reaction closely resembles that of the anaphylactic response of a sensitive uterus. The peculiar feature of the reaction is that having once responded to a dose of a venom, the plain muscle can no longer respond either to the same dose of that venom or to an equivalent stimulant dose of another venom of the group.

We have made no attempt to determine the limiting dose which with either of these venoms causes a maximal contraction of the isolated guinea-pig uterus. The venom of *Oxyuranus maclennani* is more potent than that of *Pseudechis scutellatus*. With the former a maximal response was obtained with 1 in 500,000; with the latter there was no response with 1 in 1,000,000, but a maximal response with 1 in 250,000. The denatured sample of *Oxyuranus* venom also caused a maximal response in 1 in 250,000.

CONCLUSIONS.

1. We have investigated three samples of snake venom, two unquestionably from *Oxyuranus maclennani* Kinghorn, and one which is almost certainly the venom of *Pseudechis scutellatus* Peters.

2. The venom of *Oxyuranus* injected subcutaneously is lethal to monkeys in doses of the order of 1·0 mgm. per kilo, to rabbits in those of 0·5 mgm. per kilo, to guinea-pigs in those of 0·08 mgm. per kilo, and to rats in doses of the

[2] Submitted for publication to the British Journal of Exp. Pathology.

order of 0·6 mgm. per kilo. Injected intravenously it is lethal to rabbits in doses of the order of 0·1 mgm. per kilo.

3. The venom contains "thrombase," and causes intravascular coagulation when injected in sufficient dosage. It coagulates citrated plasma *in vitro*.

4. It is only feebly haemolytic, but is powerfully neurotoxic, death in most animals being due to this activity of the venom.

5. It has a slight immediate depressor effect on the blood pressure, and finally causes death by respiratory failure. It diminishes the output of the heart.

6. The venom presumed to be that of *Pseudechis scutellatus* injected subcutaneously is lethal to rabbits in doses of the order of 2–3 mgm. per kilo, to guinea-pigs in doses of from 1·5–2·0 mgm per kilo, and to rats of 4–8 mgm. per kilo. Injected intravenously in rabbits it kills in doses of about 0·7 mgm. per kilo.

7. The venom has powerful anticoagulant and haemolytic activities, but is only feebly neurotoxic.

8. Its effects on the circulation in rabbits are in the main secondary to its haemolytic action.

9. Both these venoms have a stimulant action on isolated plain muscle.

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THE ABSORPTION OF ULTRA-VIOLET LIGHT BY OXYHAEMOGLOBIN AND BY SOME OF ITS DERIVATIVES

by

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A number of workers have endeavoured to extend the knowledge of the absorption spectra of the blood pigment and its immediate derivatives into the ultra-violet region. Soret (10), using diluted blood, detected an absorption band in three of the pigments, but wrongly assigned the band in one of them to the serum protein. Gamgee (2) detected a band in the region of 4100 A.U.[1] In 1909 Rost, Franz and Heise (8) photographed as far as 3900 A.U., the absorption spectra of oxyhaemoglobin and of numerous derivatives including certain porphyrins. Lewin, Miethe and Stenger (7) placed the head of the main band at 4150 A.U. Schumm (10) considered that it is at 4135–4140 A.U. Schumm summarizes the work to the date of publication. The work referred to was done without the help of a photometer, and hence only approximate estimations of the relative intensities of the absorption bands could be made.

The work described in this paper was carried out in order to ascertain the differences in the molecular absorption curves of various substances closely related to haemoglobin due to the differences in their constitution. The proteins were prepared in Melbourne and sent to Adelaide, where they were photographed with a Hilger size C ultra-violet spectrograph in conjunction with a Judd Lewis sector photometer. A condensed spark between iron electrodes in air was used as a source of ultra-violet light. Several kinds of plates were used, including Hauff Flavin for the longer wave-lengths, but Hauff ultra-rapid H. & D. 240 were preferred, particularly for determinations involving the shorter wave-lengths. The wave-length scale of the instrument was calibrated against the standard iron spark spectrum.

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[1] A.U.=Ångström Unit.

The proteins were prepared and stored in a refrigerator. During transport they were surrounded with crushed ice in a vacuum flask.

Ox blood was used as the source for all the substances used in these experiments. Ox oxyhaemoglobin possesses two advantages: it turns to methaemoglobin only comparatively slowly, and its globin can be readily prepared in fair concentration in a stable condition. The oxyhaemoglobin solution was prepared from well-washed corpuscles by the method of Adair (1), and dialyzed until free from salts. It was transported in strong solution, and diluted to the required concentration just before its spectrum was photographed. Small amounts of the strong solution were converted into methaemoglobin by potassium ferricyanide, and into CO-haemoglobin by pure carbon monoxide, in each case immediately before dilution and photography. For alkaline methaemoglobin 0·01 N ammonia was used as the diluting liquid instead of water. The haemoglobin was reduced with 0·8 cc. 10% sodium hydrosulphite and 0·8 cc. 5% sodium carbonate in 100 cc. solution. This was the minimum amount that was found to ensure complete reduction during the experiment. Unfortunately even this small amount of salts completely absorbed the light of the shorter wavelengths. It is hoped to study this substance using a special cell from which the oxygen can be exhausted by a pump. The (native) globin was prepared by the method of Hill and Holden (5). The remaining denatured globin was removed by half-saturation with ammonium sulphate. The globin was precipitated with ammonium sulphate, redissolved, and dialyzed until free from salts. The denatured globin was prepared from its precipitate, which occurs during the preparation of globin. It was dissolved in dilute hydrochloric acid, and the remaining pigment was extracted with alcohol and ether. It was precipitated by bringing to its isoelectric point, and washed successively with water, alcohol, and ether. The solution was made by dissolving a weighed amount in 0·02 N sulphuric acid. The haemin was made from ox corpuscles by the method of Schalpejew (9). The crystals were washed alternately with strong hydrochloric acid and 1% acetic acid until no protein could be detected in the acetic acid. They were then dried in a vacuum desiccator. They were not washed with organic solvents, as these have a tendency to spoil their appearance.

The alkaline haematin was made by dissolving 1·00 mgm. of haemin in 0·1 cc. 0·5 N sodium hydroxide solution, and diluting to 100 cc. with distilled water. The alcoholic alkaline haematin solution was prepared by adding to 1.00 mgm. of haemin dissolved in 0·1 cc. 0·5 N sodium hydroxide solution 0·5 cc. water, and making up to 100 cc. with absolute alcohol. The solution of acid haematin in alcohol was similarly prepared, but after most of the alcohol had been added 0·1 cc. concentrated aqueous hydrochloric acid (31·52% HCl) was added before making up to the mark.

The solution of acid haematin in water contained 0·005% gelatin as a protective colloid. In every case in which any additional reagent was in the

solution whose absorption was to be determined the same reagent in equal concentration was in the control tube.

Table 1 shows the positions of the bands observed. The other tables contain the data on which the curves are based. These data are those nearest the mean curve for all the data for each substance. The total number of points actually determined in defining the curve varied from 100 to 250, according to the complexity of the absorption curve. The tables are inserted with a view to assisting other workers to form some idea of the degree of regularity that can be expected with this instrument. In plotting the curves we have followed Henri (3) in making the abscissae proportional to the differences in frequency and not to differences in wave-length. The values for the proteins have been calculated on the basis of an equivalent weight for oxyhaemoglobin of 16,700. The molecular weight of haemin was taken as 652. The curves for the two classes of substances derived respectively from these two pigments are therefore directly comparable. The equivalent weight of globin was taken as 16050, so that the curves for it and for denatured globin might be comparable with the rest.

The chief points of interest revealed by a comparison of the curves are as follows: The curves for oxyhaemoglobin and CO-haemoglobin are very similar, but the bands of the latter are rather more intense than those of the former. In methaemoglobin the second band entirely disappears. In alkaline methaemoglobin the main band becomes shorter and broader, and more like that of alkaline haematin, while both the other bands are absent. There does not, as yet, appear to be any definite relationship between the valency of the iron atom in the pigment and its ultra-violet absorption spectrum. The profound influence of the state of aggregation of acid haematin on its absorption band is in accordance with the greater intensity of its band in the red portion of the spectrum when dissolved in an organic solvent. Keilin (6) discusses the relation of the absorption spectrum of a pigment to its physical state, but in the instances he quotes the wave-length of the head of the band was increased by association. The ultra-violet absorption spectra of alkaline and of acid haematin in water appear to be dependent on the pH and on the presence of electrolytes. They are also much less sharply defined than those of the other pigments examined. There is little, if any, difference between the absorption spectra of native and of denatured globin, in spite of considerable differences in their optical activities.

Similar results have been obtained in the case of other proteins where a similar comparison was made. In these cases it would seem questionable whether there is justification for applying Drude's equation to proteins, at least in the simple form proposed by Hewitt (4). For this equation to be applicable the portions of the molecule causing the rotation and the absorption must be near together. There is, however, no evidence that such is the case in the proteins under consideration.

EXPERIMENTAL DATA.

λ.A.U. is the wave-length in Angstrom units.

ν is the frequency per second.

ϵ is the equivalent absorption coefficient.

$\frac{\epsilon_H}{\epsilon_F}$ is the ratio of the co-efficients for the head of a band and the next position of minimum absorption of a shorter wave-length.

ϵ is calculated from the equation

$$\epsilon = \log \frac{I_0}{I} \times \frac{1}{c.d}$$

where I_0 is intensity of incident light.

I is intensity of transmitted light.

c is the concentration in gramme equivalents per litre.

d is the depth of liquid in centimetres.

In the figures the abscissae at the bottom represent $\nu 10^{-12}$

The abscissae marked along the top represent λ.A.U.

The ordinates represent $\epsilon 10^{-3}$, except in the cases of figures 10 and 11, where they represent $\epsilon 10^{-2}$.

In figure 2 a graph of the absorption curve of ox globin on the same scale as that of methaemoglobin and of alkaline methaemoglobin is added for purposes of comparison.

On figure 4 the main bands of haemoglobin and of oxyhaemoglobin are compared with that of haematin, while the bands of native and denatured globin are shown on the same diagram, but with an ordinate scale of $\epsilon = 10^{-2}$.

TABLE 1.

Substance.	Head.			Foot.				Table.	Figure.
	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	ϵ_H / ϵ_F		
Oxyhaemoglobin	4115	729	125	3690	813	27.1	4.61	2	1
	3495	858	31.3	3115	963	16.7	1.87		
	2810	1068	37.6	2560	1172	27.1	1.38		
Haemoglobin	4295	698	134					3	4
C.O. Haemoglobin	4179	720	154	3648	822	14.6	10.5	4	1
	3405	881	25.0	3048	971	8.35	3.0		
	2745	1093	33.3	2505	1197	20.9	1.6		
Methaemoglobin	4045	742	134	3100	972	16.7	8.0		
	2686	1117	33.4	2525	1188	20.9	1.6	5	2
Alkaline Methaemoglobin	4106	730	71	3073	976	6.96	10.2	6	2
Alkaline Haematin in water	3847	780	48.9	2979	1007	13.0	3.8		
	2535	1183	21.2					7	3
Alkaline Haematin in alcohol	4025	745	79.5	2984	1005	16.3	4.9		
	2535	1207	32.6					8	3
Acid Haematin in water	3793	790	45.6	2964	1012	13.0	3.5	9	3
Acid Haematin in alcohol	3987	753	151	3073	976	8.1	18.6	10	3
			$\epsilon 10^{-2}$			$\epsilon 10^{-2}$			
Globin	2790	1075	134	2515	1193	66.9	1.9	11	4
Denatured Globin	2740	1095	136	2505	1198	60.6	2.2	12	4

TABLE 2.

Oxyhaemoglobin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4800	625	8.35	3985	753	66.8	3025	992	20.9
4700	638	10.4	3935	763	58.4	2995	1001	23.0
4600	652	12.5	3915	766	45.9	2935	1022	25.0
4480	670	14.6	3845	780	35.5	2880	1042	29.2
4375	685	18.8	3825	784	31.3	2835	1058	33.4
4315	695	27.1	3790	792	27.1	2805	1069	37.6
4295	698	31.3	3765	797	25.0	2765	1085	35.5
4285	700	45.9	3705	810	23.0	2695	1113	33.4
4255	705	66.8	3600	833	25.0	2665	1126	31.3
4235	708	83.5	3565	841	29.2	2625	1143	29.2
4205	713	100	3495	858	31.3	2570	1167	27.1
4195	715	109	3465	866	29.2	2525	1188	27.1
4140	724	117	3415	879	29.2	2485	1207	35.5
4130	726	125	3335	900	27.1	2450	1225	39.7
4100	732	125	3295	910	25.0	2412	1244	55.7
4080	735	117	3235	927	23.0	2385	1258	62.6
4055	740	109	3185	942	20.9	2365	1268	69.6
4050	741	100	3125	960	17.4	2345	1279	76.5
4035	743	83.5	3080	974	18.8			

TABLE 3.

Haemoglobin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4504	666	16.7	4349	690	109	4189	716	100
4478	670	25.0	4325	694	125	4160	721	91.9
4436	676	41.7	4295	698	134	4130	726	83.5
4404	681	58.4	4276	701	125	4101	731	75.1
4394	683	75.1	4237	708	117	4053	740	66.8
4373	686	91.9	4218	711	109			

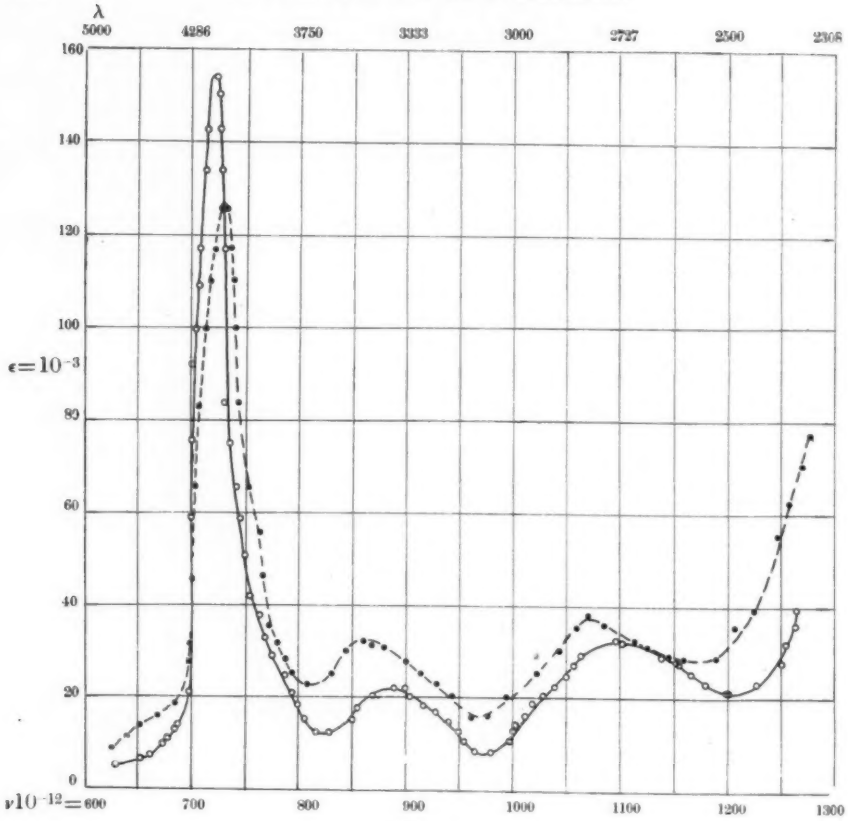

Fig. 1.

Oxyhaemoglobin —●—●—
Carboxyhaemoglobin —o—o—

TABLE 4.

CO-Haemoglobin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4750	632	5.22	4276	701	109	4082	735	75.1
4600	652	6.26	4237	708	109	4082	735	109
4540	661	7.31	4228	709	117	4062	739	66.8
4478	670	9.39	4208	713	134	4045	742	58.4
4425	678	11.48	4199	715	142	4015	747	50.1
4399	682	12.52	4179	720	154	3975	755	41.7
4373	686	14.61	4150	723	150	3945	760	37.6
4325	694	20.9	4140	725	142	3915	766	33.4
4315	695	58.4	4130	726	134	3886	772	29.2
4300	698	39.7	4121	728	125	3847	780	25.0
4295	698	75.1	4111	730	117	3801	789	20.9
4286	700	91.8	4101	731	83.5	3753	799	18.8

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
3715	807	16.7	3168	947	12.5	2720	1103	31.3
3677	816	14.6	3143	954	10.44	2641	1136	29.2
3618	829	14.6	3123	961	8.35	2607	1151	27.8
3536	848	16.7	3054	982	8.35	2602	1153	27.1
3506	856	18.8	3024	992	10.44	2570	1167	25.0
3456	868	20.9	3004	999	12.52	2545	1179	23.0
3426	876	23.0	2984	1005	14.6	2510	1195	20.9
3405	881	25.0	2969	1010	16.7	2500	1200	20.9
3375	889	23.0	2949	1017	18.8	2440	1229	23.0
3318	904	20.9	2919	1028	20.9	2415	1242	25.0
3293	911	18.8	2889	1038	23.0	2405	1247	27.1
3232	928	18.8	2864	1047	25.0	2390	1255	31.3
3232	928	16.7	2839	1057	27.1	2380	1260	35.5
3207	935	16.7	2819	1064	29.2	2370	1266	39.7
3192	939	14.6	2745	1093	33.3			

TABLE 5.

Methaemoglobin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4276	701	16.7	3637	825	31.3	2616	1147	29.2
4228	709	41.7	3582	837	27.1	2597	1155	27.1
4218	711	48.0	3546	846	25.0	2583	1161	25.0
4199	714	58.4	3506	856	22.9	2565	1170	22.9
4169	720	75.1	3456	868	20.9	2525	1188	20.9
4140	725	91.8	3375	889	18.8	2485	1207	22.9
4121	728	108.5	3242	925	16.7	2470	1215	25.0
4101	731	117	3083	973	16.7	2455	1222	27.1
4082	735	125	3014	995	16.7	2440	1229	29.2
4045	742	134	2934	1022	16.7	2425	1237	31.3
4005	749	125	2819	1064	18.8	2420	1240	33.4
3985	753	117	2785	1077	20.9	2410	1245	35.5
3945	760	100	2765	1085	22.9	2405	1247	37.6
3925	764	91.8	2755	1089	25.0	2400	1250	39.7
3905	768	83.5	2745	1093	27.1	2390	1255	41.7
3827	784	66.8	2735	1097	29.2	2385	1257	43.8
3780	794	48.0	2725	1101	31.3	2380	1260	45.9
3762	797	43.8	2710	1107	33.4	2375	1263	48.0
3744	801	39.7	2661	1127	33.4			
3706	809	35.5	2641	1136	31.3			

TABLE 6.

Alkaline Methaemoglobin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4478	670	6.96	3780	794	33.4	2904	1033	16.7
4373	686	13.9	3706	809	31.3	2894	1037	18.9
4325	694	20.9	3648	822	29.2	2869	1046	20.9
4305	697	27.8	3574	839	27.1	2844	1055	22.9
4276	701	37.6	3466	866	25.0	2809	1068	25.0
4252	706	48.7	3375	889	22.9	2785	1077	27.1
4228	709	55.7	3308	907	20.9	2775	1081	29.2
4179	718	62.6	3382	914	18.8	2745	1093	31.3
4155	722	69.6	3242	925	16.7	2725	1101	33.4
4155	722	66.8	3202	937	14.6	2695	1113	35.5
4130	726	71.0	3173	945	12.5	2666	1125	37.6
4082	735	71.0	3143	954	10.4	2621	1145	39.7
4035	743	66.8	3103	967	6.96	2580	1163	41.7
3985	753	62.6	3058	981	8.35	2445	1227	62.6
3915	766	55.7	3034	989	8.35	2420	1240	66.8
3876	774	48.7	2984	1005	10.4	2370	1266	71.0
3837	782	39.7	2954	1016	12.5			
3801	789	35.5	2929	1024	14.6			

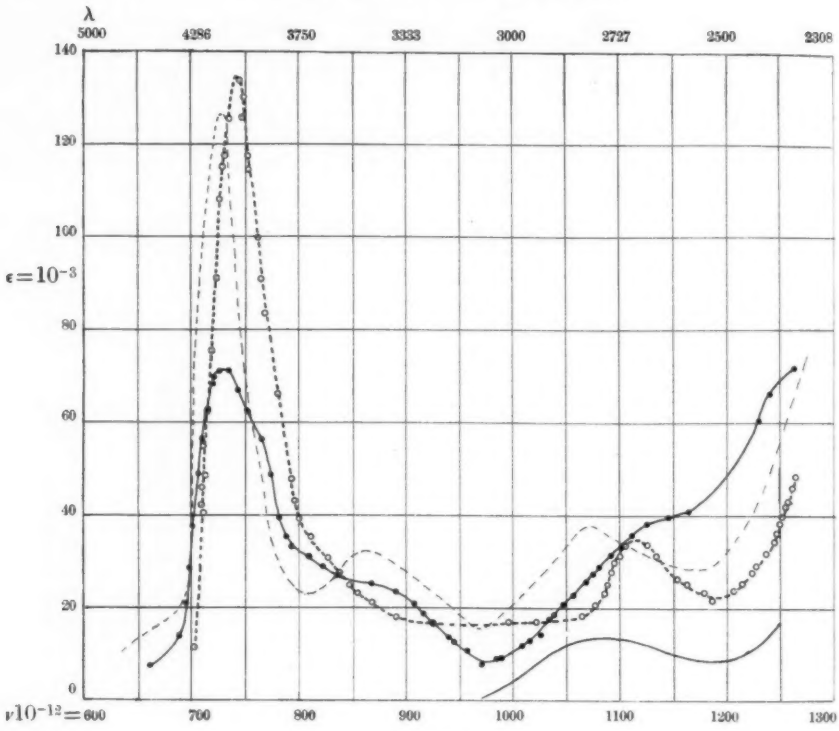

Fig. 2.

Methaemoglobin - - -o- - -o- - -
Alkaline Methaemoglobin —●—●—
Oxyhaemoglobin — — — —
Globin (Native) plotted on same scale of ordinates ———

TABLE 7.

Alkaline Haematin in Water.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4373	686	13.0	3896	770	48.9	3123	961	16.3
4276	701	14.7	3801	789	48.9	2979	1007	13.0
4155	722	19.6	3735	803	47.6	2864	1047	14.7
4106	731	22.8	3609	831	42.4	2795	1073	16.3
4045	742	27.5	3506	856	37.1	2676	1121	19.6
4035	743	32.6	3476	863	32.6	2535	1183	21.2
4005	749	36.1	3375	889	26.1	2415	1242	19.6
3945	760	42.4	3252	922	22.8	2355	1278	17.9
3905	768	46.6	3178	944	19.6	2225	1348	16.3

TABLE 8.

Alkaline Haematin in Alcohol.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4505	666	14.7	3955	759	75.0	2984	1005	16.3
4373	686	17.9	3935	762	73.0	2914	1029	17.9
4300	698	21.2	3876	774	68.5	2969	1010	17.1
4276	701	22.8	3762	797	60.3	2909	1031	19.6
4252	706	26.1	3696	812	57.0	2854	1051	22.8
4208	713	34.2	3627	827	53.8	2755	1089	26.1
4179	718	39.1	3546	846	48.9	2636	1138	31.0
4160	721	44.0	3476	863	42.4	2570	1167	32.6
4135	726	50.5	3426	876	39.1	2535	1183	32.6
4106	731	61.9	3354	894	35.9	2485	1207	32.6
4087	734	68.5	3303	908	32.6	2470	1215	31.0
4058	739	75.0	3267	918	29.3	2445	1227	29.3
4049	741	76.9	3197	938	26.1	2420	1240	26.1
4040	743	78.2	3123	961	22.8	2390	1255	22.8
4025	745	79.5	3093	970	21.2	2350	1276	17.9
3985	753	78.2	3054	982	19.6			
3975	755	76.9	2999	1000	17.9			

TABLE 9.

Acid Haematin in Water.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4750	632	6.52	3618	829	42.4	2914	1029	13.0
4600	655	9.78	3566	841	39.1	2814	1066	14.6
4478	670	13.0	3516	853	32.6	2730	1099	16.3
4373	686	16.3	3466	866	26.1	2631	1140	17.9
4276	701	19.6	3416	878	22.8	2583	1161	19.6
4228	709	22.8	3328	901	21.2	2540	1181	22.8
4082	734	32.6	3282	914	19.6	2515	1193	26.1
4035	743	39.1	3232	928	17.9	2500	1200	29.3
3935	762	42.4	3158	950	16.3	2480	1210	32.6
3861	778	45.6	3123	961	14.6			
3735	803	45.6	3024	992	13.0			

TABLE 10.

Acid Haematin in Alcohol.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
4276	701	6.52	4000	750	151	3436	873	26.1
4223	710	14.3	3975	755	151	3385	886	22.8
4218	711	19.6	3955	759	149	3339	899	19.6
4179	718	32.6	3954	760	146	3303	908	16.3
4169	720	35.9	3935	761	141	3261	920	14.7
4160	721	45.6	3930	763	137	3222	931	13.0
4140	725	58.7	3925	764	114	3158	950	11.4
4121	728	71.7	3915	766	111	3118	962	9.8
4101	731	84.8	3900	769	97.8	3073	976	8.1
4066	738	97.8	3886	772	84.8	3029	990	9.8
4060	739	111	3857	778	78.2	2964	1012	11.4
4055	740	117	3801	789	71.7	2894	1037	13.0
4053	740	121	3744	801	58.7	2814	1066	14.7
4049	741	124	3682	815	45.6	2725	1101	16.3
4035	743	133	3658	820	42.4	2666	1125	19.6
4030	744	137	3627	827	39.1	2570	1167	22.8
4025	745	141	3600	833	35.9	2545	1179	26.1
4015	747	149	3476	863	29.3			

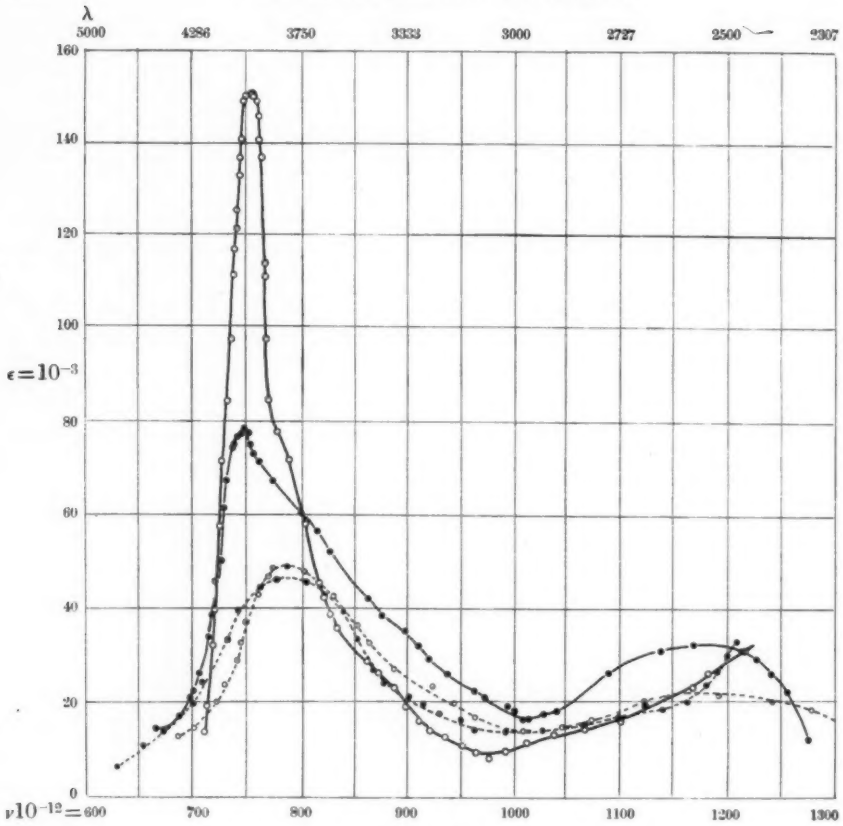

Fig. 3.

Alkaline Haematin in alcohol —●—●— in water - - -o- - -o- - -
Acid Haematin in alcohol —o—o— in water - - -●- - -●- - -

TABLE 11.

Globin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
3093	970	13.4	2859	1049	120	2520	1190	73.5
3029	990	26.7	2824	1062	134	2500	1200	66.9
3014	995	26.7	2765	1085	134	2480	1210	80.2
2984	1005	40.1	2745	1093	134	2465	1217	80.2
2959	1014	53.5	2671	1124	120	2455	1222	93.6
2954	1016	53.5	2666	1125	120	2450	1224	93.6
2949	1017	66.9	2636	1138	107	2440	1229	107
2934	1022	80.2	2616	1147	107	2435	1232	107
2914	1029	93.6	2587	1160	93.6	2425	1237	120
2904	1033	93.6	2580	1163	80.2	2415	1242	134
2884	1040	107	2550	1176	80.2	2410	1245	147
2864	1047	120	2530	1186	66.9	2405	1247	160

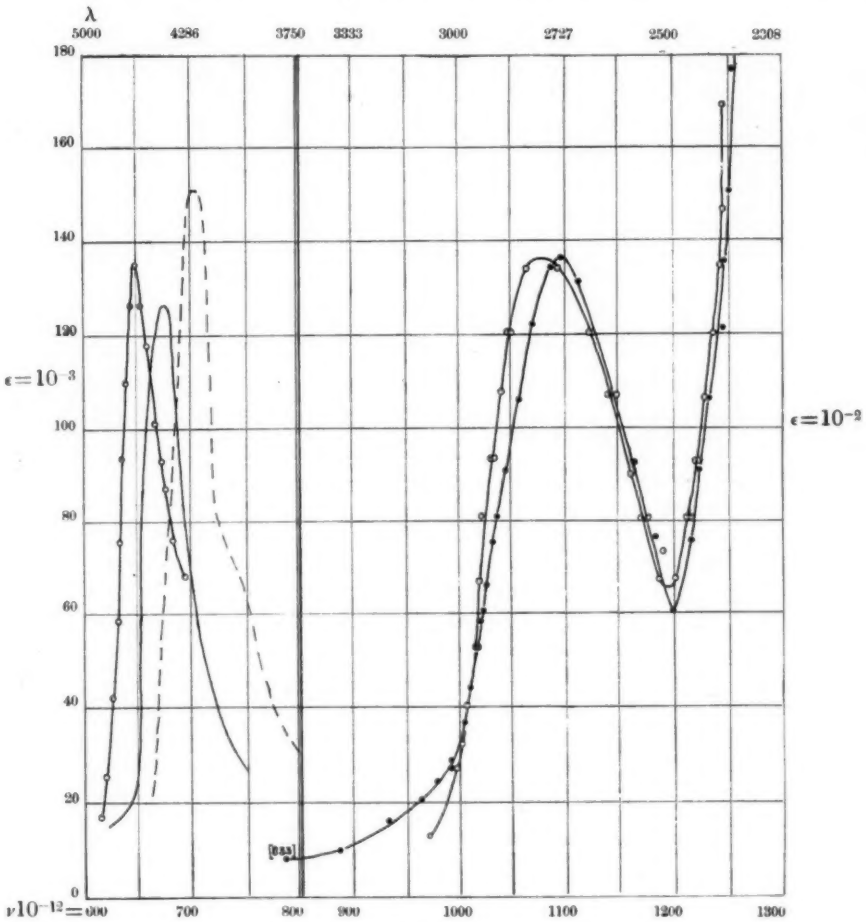

Fig. 4.

Reduced Haemoglobin —o—o—
Oxyhaemoglobin ———
Acid Haematin in Alcohol — — —
Native Globin —o—o—
Denatured Globin —●—●—
Ordinates $\epsilon=10^{-2}$

TABLE 12.

Denatured Globin.

λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$	λ.A.U.	$\nu 10^{-12}$	$\epsilon 10^{-3}$
3600	833	7.30	2934	1022	58.4	2587	1160	90.8
3385	886	9.20	2929	1024	60.6	2540	1181	75.7
3232	928	16.05	2924	1026	65.7	2505	1198	60.6
3123	961	20.1	2909	1031	73.0	2470	1215	75.7
3068	978	24.1	2904	1033	75.7	2450	1225	90.8
3029	990	28.1	2899	1035	80.3	2430	1235	106
2999	1000	32.1	2874	1044	90.8	2410	1245	121
2994	1002	36.1	2844	1055	106	2403	1248	136
2984	1005	40.1	2804	1070	121.1	2395	1253	151
2974	1009	44.1	2740	1095	136	2390	1255	167
2959	1014	45.4	2695	1113	121	2380	1260	182
2959	1014	48.1	2631	1140	106			

SUMMARY.

Numerical data and graphs are presented, showing the ultra-violet absorption spectra of haemoglobin, of some of its immediate derivatives, and of haematin and globin.

The main band of the pigments lies in the neighbourhood of 4000 A.U. This typical band is due to the haematin molecule. Its position and intensity are not related to the valency of the iron atom. For globin the main band is at 2790 A.U.

The absorption spectra of globin and denatured globin are practically identical as far as 2400 A.U.

The absorption curves of acid and of alkaline haematin in alcohol differ from those of these substances in water.

Table 1 indicates the main features of the various absorption curves determined. The detailed numerical data are given in the succeeding tables.

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STUDIES IN THE REACTION OF URINE

by

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The Alkaline Tide.

The alkaline tide, which occurs in urine from time to time throughout the day, is a fluctuation of its reaction towards relative or absolute alkalinity. It may be investigated by estimating the titratable acidity of the urine, and/or the hydrogen ion concentration. The writer accepts as evidence of an alkaline tide, in samples of urine taken hourly, a rise of 1·0 pH unit in reaction as compared with that of the preceding sample, or an increase of 0·5–1·0 pH unit in each of two successive samples as compared with the pH of the urine collected one hour before the first of these samples. This criterion is rather more rigid than that of Munford and Hubbard (1).

The Cause of the Alkaline Tide.

Bence Jones, in 1845, attributed the alkaline tide to gastric acid-secretion, since it occurs 3–4 hours after a meal. Leathes (2) denies this explanation, stating that a tide is unusual after the midday meal, and may occur in normal persons although no breakfast be taken. He suggests that the tide is due to the deepening of respiration which occurs in the morning, with consequent removal of CO_2 from the body, a view which he supports by the observation that prolonged voluntary hyperpnoea produces a more alkaline urine. Campbell (3), however, states that the tide is usual after both morning and afternoon meals, and the afternoon tide is not infrequently greater than the morning one. Though an alkaline tide may occur though no breakfast be taken, yet this does not constitute evidence that no hydrochloric acid is being produced by the stomach; acid-secretion due to habit and psychical causes cannot be ignored. Even in normally-breathing subjects, who are not hyperpnoeic, an alkaline tide occurs. Stressing the correlation in time between the maximum acidity of the stomach and the maximum alkaline tide, i.e., 3–4 hours after a meal, he accepts the gastric-secretion view.

Dodds (4) describes an alkaline tide following the first meal of the day, and lasting 1–2 hours; thereafter an acid tide, lasting 2–3 hours, and obscuring

the afternoon alkalinity following the midday meal. An afternoon tide was produced only if no breakfast had been taken. Dodds accepts the gastric-acid-secretion as causing the alkaline tide, and the alkaline pancreatic-secretion as causing the acid tide.

Factors Influencing the Alkaline Tide.

The various authorities diverge widely, but the following views of the causation of the alkaline tide are advanced.

A. *Gastric Acidity.* If H ions be removed from the system as gastric HCl, it would seem necessary that corresponding excretion of OH ions occur to conserve the constant pH of the blood. Since the kidney is the great, possibly the only, eliminator of alkali (Meyer), such OH excretion may well take the form of an alkalinity of the urine. The writer has, however, as yet seen no attempt to ascertain the pH of the blood in relation to gastric acid-secretion and the occurrence of an alkaline tide in the urine.

The views stated in the literature connecting gastric acidity with the alkaline tide are, however, so contradictory that it has been found well worth conducting personal investigations, which will appear in the body of the paper.

B. *Diet.* Most foodstuffs are not so definitely acid or alkaline as to influence directly the reaction of the urine. But, since the time of Beaumont (5) we have recognized that varying foodstuffs excite very different amounts of gastric acid-secretion, or, as we may now say, cause different outpourings of H ions from the system. A food causing a large acid output might thus be expected to cause a big compensatory hydroxyl-ion output, i.e., a big alkaline tide in the urine. To this point we will later recur.

C. *Respiration.* Leathes (2), owing to the alkalinity of the urine following hyperpnoea, and the acidity of the night urine (which he attributes to lessened ventilation of CO_2 during sleep), holds that respiration is the factor ruling the alkaline tide. Campbell (3) has denied this view. Palmer and Van Slyke (6) state that the urine of normal man approaches the alkalinity of blood when the plasma-bicarbonate CO_2 exceeds 71 $\pm$ 5 vols. %. Gamble (7) holds that the CO_2 tension of the urine depends on that of the blood plasma. Collip and Backus (8) find that hyperpnoea raises the blood pH, reduces the alveolar CO_2 tension and the combining value of the plasma for CO_2, and renders the urine less acid and with greater phosphate content.

Watson (9) finds the alveolar CO_2 tension high on waking, as described by Leathes, and falling later in the morning. Dodds (10) states that it rises after a meal and falls later. In one patient, partially gastrectomized, this rise failed to occur, and Dodds believes that connection exists between CO_2 tension and gastric HCl-secretion. Watson found no rise of CO_2 tension in patients showing the alkaline tide during a morning fast, and thereby concludes that

there could have been no psychical secretion of HCl, i.e., the alkaline tide depends on respiration, not upon digestion.

In the experiments of Leathes the reaction of the blood was at equilibrium; CO_2 output, as in hyperpnoea, then occurred without any new formation of CO_2. The resulting loss of H ions would surely tend to alkalinity of the blood, and compensatory excretion of OH ions, giving an alkaline urine. But after a meal, as in the experiments of Watson and Dodds, a huge new formation of CO_2 must occur, giving a high alveolar tension and a lower pH of the blood. The excess CO_2 must therefore be rapidly excreted by the lungs, as we know occurs. The production and excretion of this CO_2 is quite independent of increased secretion of alkali in the urine; one might rather expect holding-up of hydroxyl from the urine till the excess CO_2 had been breathed away. (Might this explain the "acid tide" of Campbell, occurring in the first 1–2 hours after a meal?) Neither can one connect secretion of gastric HCl with CO_2 output by the lungs. Acid secretion would tend to alkalinize the blood, and should rather be followed by holding-up of acid from the urine, than by a further excretion of acid ions resulting from loss of CO_2. Absence of increased CO_2 output during fasting is surely no proof that no acid-secretion is proceeding in the stomach.

Gas-exchange and urinary reaction may proceed independently. Lutz (11) has shown that, in the horse and in man, an alkaline urine can be produced by a diet which does not alter the combining value of the plasma. Meyer (12) demonstrates wide variations in gas-exchange after taking certain sugars, the urinary reaction remaining appreciably unaltered. Indeed, the evidence seems to show that respiration is far from the decisive cause of the alkaline tide of urine. Capable as it is of rapidly and extensively removing H ions from the body, it probably plays some rôle, though only a subordinate one. Some personal evidence for this view will be discussed subsequently.

D. *Heat and Work.* Talbert (13) demonstrates an increased acidity of the urine following hard work or sweating. An acid urine is clinically usual in association with pyrexia and hard exercise. Talbert does not offer an explanation of his findings; nor would mere concentration appreciably affect the pH of a highly-buffered solution, as urine. It is possible that the enormously increased metabolism of exercise, with its consequent great production of CO_2, might lead, in the interest of a constant pH of the blood, to actual holding-back of hydroxyl from the urine (i.e., production of an acid urine) till the excess of CO_2 be ventilated away. Another explanation may lie in the observation of Talbert and Rosenberg (14), that sweating appreciably diminishes gastric acid-secretion. Compared, however, with the important rôle played by food in altering the urinary reaction, that of work and heat can be but small indeed.

Ammonia Excretion. Hasselbalch (15) states that pH Urine x (Ammonia Nitrogen/Total Nitrogen) = K, where K is constant. Fiske (16) denies the universal applicability of this formula, and can find no consistent relationship

between the two quantities. Howe and Hawke (17) found no connection between ammonia N_2 and urinary pH during a prolonged protein-feeding experiment. Campbell, however, states that gastric acidity and urinary ammonia-nitrogen lie in inverse ratio.

Rafflin (18) suggests, as preferable to Hasselbalch's coefficient, the following: pH/4 + Log (Ammonia N_2/Total N_2) = K, where K is constant. Polonovski and Boulanger (19) have criticized this value, but Rafflin holds that his findings approximate closely to theirs, and more closely than do computations based upon Hasselbalch's formula. Without personal experience in this field, the writer believes that NH_3-excretion is but one of the factors playing upon urinary reaction, and, as Polonovski and Boulanger state, no exact mathematical expression is possible.

The Problem for Research.

From the foregoing it will be seen necessary to investigate:

1. The constancy or otherwise of the presence of the alkaline tide in the urine of normal individuals.
2. The relation of the tide to various articles of diet.
3. The relation of the tide to the secretion of gastric hydrochloric acid.
4. The behaviour of the tide following ingestion of certain drugs.
5. The possible clinical applications of the knowledge gained. These five inter-related investigations constitute the present paper.

EXPERIMENTAL METHODS.

Subjects.

These were young and healthy men, free of clinical urinary or gastric disease.

Diet.

Mixed diet was usually chosen, as reproducing the normal conditions of life. Breakfast, 8.30 a.m., eggs, porridge or cereal, toast, butter, and tea. Luncheon, 1 p.m., soup, meat, bread, and sweet, milk or tea. No food was permitted between meals, but water and tobacco were allowed in moderation.

Collection of Urine.

This was effected hourly, care being taken to guard against loss of CO_2, which Marshall (20) has shown to greatly raise the pH of the sample, especially if already alkaline. Alveolar air has a CO_2 content proportionate to that of urine (Marshall, 20; Frederieq, 21), and by collection in an atmosphere of alveolar air much loss of CO_2 was averted. Conserving CO_2, Marshall states that the maximum pH of normal urine is 8·0; the writer's highest reading was 8·1, nearer to Marshall's value than that of Henderson (22), who did not conserve CO_2, and whose maximum alkalinity was 8·7.

Technique.

The indicators of Henderson and Palmer (23) have been now superseded by the phenolsulphonephthalein group introduced by Clark and Lubs (24). A series of these was employed at first by the writer, ranging from methyl-red to phenol-red (pH 4·6 to pH 8·6), prepared after Hatfield's modification of Gillespie's method (25). In turbid fluids, as urine may be, these fall well below the 0·1 pH-unit accuracy claimed for them. Rather than dilute the urine (for the influence of which on the pH value see the work of Myers and Muntwyler, 26), recourse was had to the quinhydrone-electrode method with which most of the present work was done, and which is probably here accurate to 0·05 pH-unit. All results have been corrected to the first decimal place in the interest of graphic representation.

SECTION I. THE ALKALINE TIDE IN NORMAL INDIVIDUALS UPON MIXED DIET.

The Normal Day Curve.

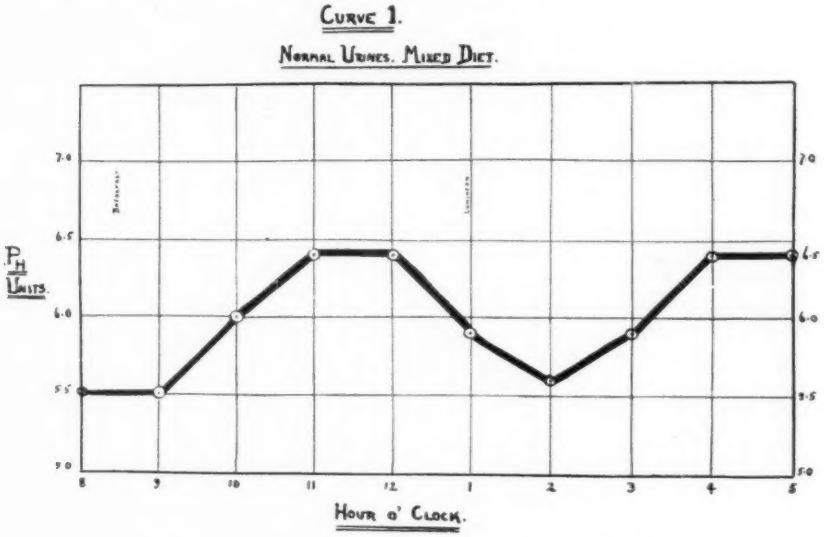
Fourteen experiments upon six subjects, between 8 a.m. and 5 p.m., were effected to ascertain the frequency of an alkaline tide in normal individuals upon a mixed diet. (See Table 1.)

TABLE 1.

Normal Day Urines. Mixed Diet. Hourly Readings.
Breakfast, 9 a.m. Luncheon, 1 p.m.

	pH.					
Hour.	14/5/28	15/5/28	17/5/28	21/5/28	22/5/28	11/6/28
8 a.m.	5.3	5.5	5.5	5.4	5.3	5.5
9 „	5.3	6.0	5.7	5.4	5.4	5.5
10 „	5.4	6.3	6.5	5.5	5.5	6.5
11 „	5.4	6.8	6.7	6.5	6.3	7.4
12 „	6.3	6.1	6.5	6.8	7.3	7.1
1 p.m.	5.7	5.7	5.7	5.8	6.4	6.9
2 „	5.4	5.7	5.3	5.5	5.7	5.9
3 „	5.9	6.1	5.5	5.6	5.9	6.6
4 „	6.8	7.0	6.5	5.9	7.1	6.5
5 „	6.0	7.0	6.3	6.0	7.2	6.0
6 „	—	6.5	5.7	5.5	6.9	—
Hour.	23/10/28	25/10/28	?	?	2/11/28	13/11/28
8 a.m.	—	—	5.2	—	—	5.4
9 „	—	—	5.2	—	—	5.3
10 „	6.9	6.7	5.5	6.1	5.9	5.3
11 „	7.5	7.3	6.4	6.0	6.6	5.5
12 „	7.3	7.3	6.3	5.7	6.3	5.7
1 p.m.	6.7	6.7	5.2	5.3	6.0	6.3
2 „	6.6	6.8	5.2	5.6	5.5	5.5
3 „	6.3	6.3	5.3	6.2	6.1	5.4
4 „	6.7	7.0	6.3	6.6	6.6	5.4
5 „	6.8	7.8	6.5	6.3	6.6	6.2
6 „	6.6	—	—	—	—	5.9

These figures are reduced to a mean curve in Curve 1. A morning and afternoon tide (both) are present in eleven cases; an afternoon tide is absent in one case only (21/5/28) in the presence of a morning tide, and the morning tide is never absent where an afternoon tide is present. Except for 21/5/28, in no experiment does a great morning tide imply the absence of the afternoon tide. Usually well-defined tides occur both morning and afternoon; the picture rather resembling that of Campbell (3) than that of Dodds (4). A brief acid phase soon after a meal is the nearest approach to an "acid tide," again resembling Campbell's findings rather than Dodds'.



Campbell attributes this acidity to secretion of alkaline saliva; but saliva is not very abundant. Dodds (4) attributes it to pancreatic flow. Now, the rate of secretion of the pancreas depends on the acidity of the stomach juice (Pavlov, 27), which is maximal three hours after a meal, i.e., later than the "acid tide." Starling (28) states, too, that the maximum pancreatic flow is reached 2–3 hours after food. Reference to Table 10 will show that the "acid tide" occurs when the gastric HCl is at a low level, but this will not explain the cases where the acid tide actually reaches a pH value a fraction of a unit below the fasting level.

Anomalous Curves.

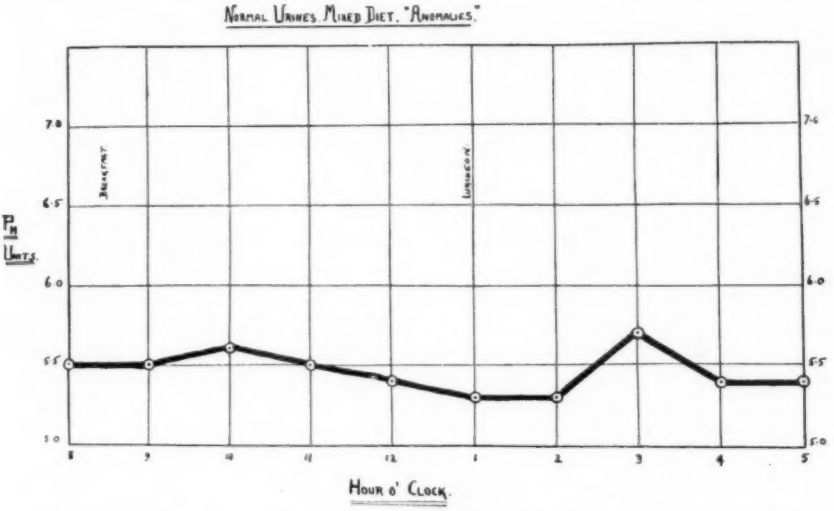
In two experiments, detailed in Table 2, and "averaged" in Curve 2, no alkaline tide was present at all.

TABLE 2.

Normal Urine. Mixed Diet. Anomalous Curves.

Hour.	pH. 30/7/28	pH. ?	Hour.	pH. 30/7/28	pH. ?
8 a.m.	5·4	5·5	1 p.m.	5·3	5·2
9 „	5·2	5·7	2 „	5·1	5·4
10 „	5·4	5·7	3 „	5·9	5·5
11 „	5·4	5·5	4 „	5·4	5·3
12 „	5·3	5·4	5 „	5·3	5·4

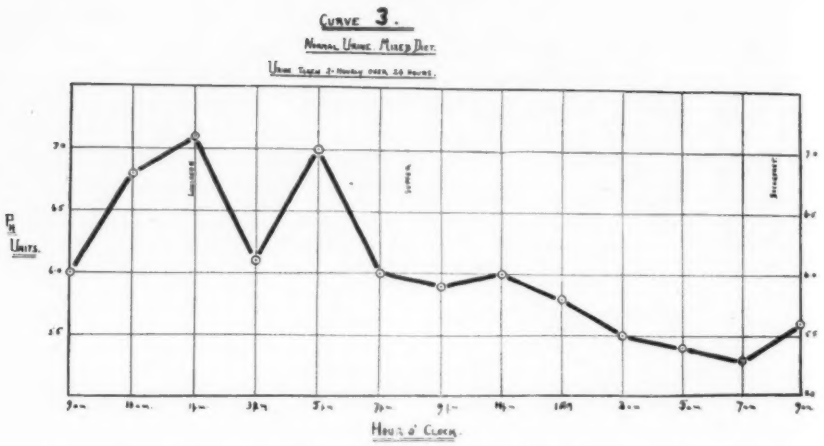
CURVE 2.



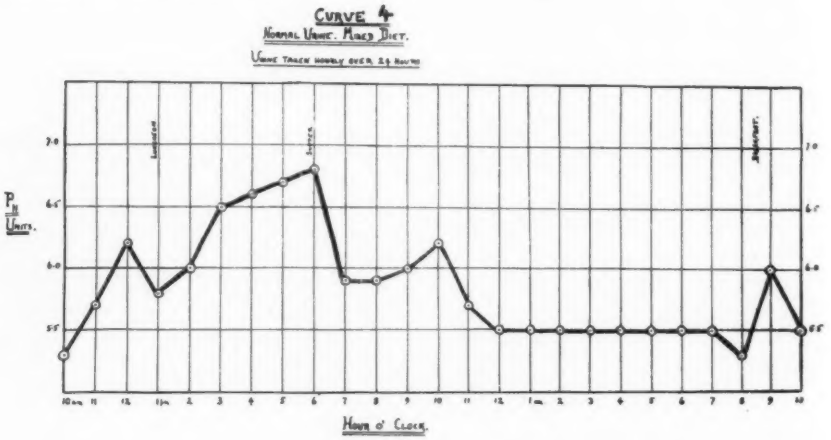
Unfortunately, this type of curve has not been encountered since facilities have existed for estimating gastric acidity on a pH basis.

The Night Urine.

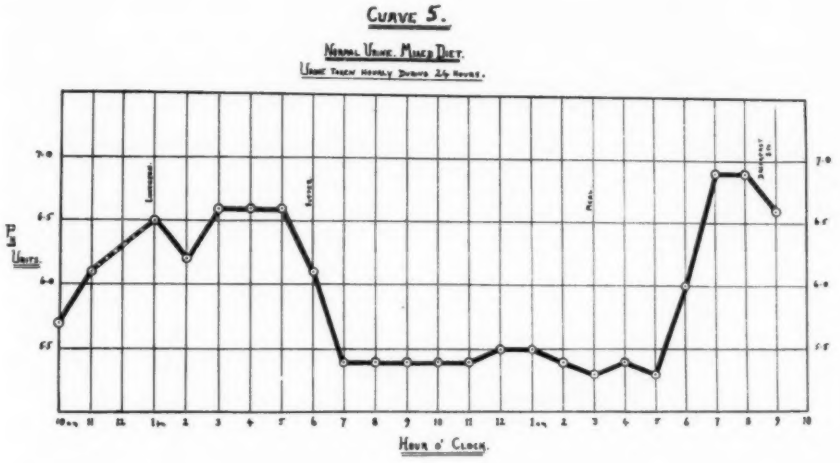
Curve 3 represents the pH chart of a normal urine taken two-hourly over 24 hours. Morning and afternoon alkaline tides are well defined. The progressive fall in pH during the hours of night has been observed by Leathes (2), who attributes it to lessened pulmonary ventilation of CO_2 during sleep. In this and the other 24-hour curves of the present paper the important difference exists that the subject was not asleep, but was awake and performing laboratory experiments. Yet the nocturnal acidity is still progressive. Now, when one is awake in a well-warmed laboratory, and performing the same experimental work as by day, the respiratory exchange must be not necessarily identical with, but very closely parallel to, that obtaining during the day. Yet the urine, which by day shows wide fluctuations in reaction, is at night consistently acid. It follows, therefore, that the consistently acid night urine is probably not due to deficient ventilation of CO_2 as Leathes suggests.



One must, however, consider the complete abstention from food over the 14-hour period, 6 p.m. to 8.30 a.m. This starvation may set up a condition of relative acidosis. Admittedly much glycogen remains stored in the liver, but work recorded in this paper (Tables 6 and 7) and by Meyer (12), tends to show that pure carbohydrate does not itself produce an alkaline tide in the urine. Moreover, the present paper seems to show definitely that the nocturnal acidity is due to starvation, since it may be abolished by taking (non-carbohydrate) food. Relative acidosis would imply a tendency to a lowered pH of the blood, and the excess of H ions may be excreted, giving an acid urine. Rothera's test for acetone was negative.



Curve 4 represents a similar 24-hour experiment, save that pH readings were taken hourly. Morning and afternoon alkaline tides and a nocturnal acidity reaching equilibrium at pH 5·5, and remaining so nearly all night, are again displayed. These are no less prominent in Curve 5, an identical experiment. Here, however, having allowed the acid level to reach equilibrium, a meal of "Bovril," toast, and tea was permitted at 3 a.m. A marked alkaline response followed, which, since "Bovril" and other meat-extractives are strong stimulants to gastric HCl-secretion, is the more likely to indicate a coincidence between gastric acidity and urinary alkalinity.



The Alkaline Tide after Supper.

In the above curves the absence of an alkaline tide after supper will be noted, except for a brief alkalinity (0·4 pH-unit for 1 hour) in Curve 4. If the tide be absent, and a normal secretion of gastric HCl be present at this time, the co-relation of gastric with urinary reaction must be abandoned.

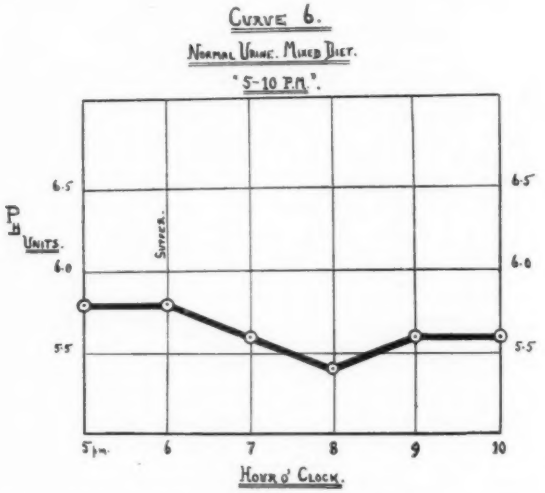
Five experiments were therefore conducted on subjects between the hours of 5 p.m. to 10 p.m. An ample supper of meat or eggs, with tea, toast, and a sweet, was allowed at 6 p.m. Breakfast and luncheon were at the usual times, and sufficiently rich in protein to call up adequate gastric acid-secretion. The findings are tabulated in Table 3.

TABLE 3.

Normal Urine. Mixed Diet. 5–10 p.m.

Hour.	23/11/28	23/11/28	19/11/28	13/12/28	13/12/28
			pH.		
5 p.m.	6.9	5.7	5.9	5.4	5.1
6 „	6.9	5.7	6.3	5.1	5.0
7 „	6.5	5.4	5.9	5.0	5.0
8 „	6.2	5.4	5.4	5.1	5.1
9 „	6.2	5.4	5.5	5.7	5.3
10 „	6.5	5.4	5.5	5.5	5.3
11 „	6.1	5.5	—	—	—

The results are "averaged" in Curve 6, the latter showing a slight alkaline phase from 9–10 p.m., which, however, does not constitute an alkaline tide. No true alkaline tide is present in any of the five experiments. It then remained, which was less easy, to ascertain the gastric acidity during the hours following the evening meal.



Two volunteers presented themselves, and a combined curve of gastric and urinary acidity was prepared, as will be described in Section 3. The results are set out in the accompanying Table 4.

TABLE 4.

Normal Urine. Mixed Diet. Combined Gastric and Urinary Reaction.

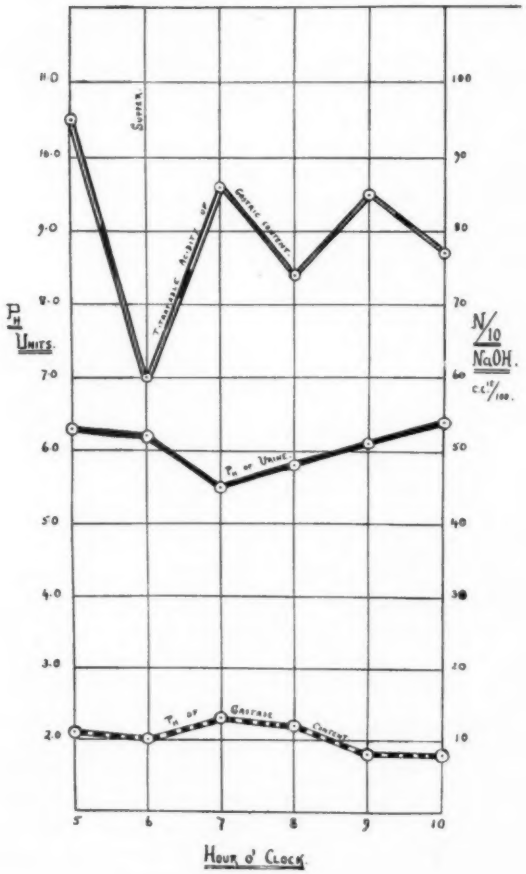
Date.	Hour.	pH of Urine.	pH Gastric Content.	Titratable Acidity, Gastric Content. (NaOH ccs. %)
20/11/28	5 p.m.	6.6	2.1	106
	6 „	6.5	1.8	66
	7 „	5.8	2.6	102
	8 „	6.4	2.8	70
	9 „	6.4	2.0	124
	10 „	6.8	2.2	80

Date.	Hour.	pH of Urine.	pP Gastric Content.	Titratable Acidity, Gastric Content. (NaOH ccs. %)
3/12/28	5 p.m.	6·1	2·2	84
	6 „	6·0	2·3	54
	7 „	5·3	2·1	70
	8 „	5·2	1·6	78
	9 „	5·9	1·6	46
	10 „	6·0	1·4	75

CURVE 7.

NORMAL URINE. MIXED DIET.

POST-SUPPER CURVE OF GASTRIC & URINARY ACIDITY.



It is unfortunate that both subjects should have shown an alkaline tide; one showing no tide would have been more informative. The experiments indicate that, though an individual reading may show a lowered gastric pH at a time when the urinary reaction might seem to call for a raised, yet in the main a phase of increased H ion concentration is present in the gastric contents at a time when a phase of alkalinity is manifest in the urine. This suggests an inverse relationship between the gastric and urinary acidity.

In Curve 7 the two experiments are reduced to a mean graph exemplifying the above-mentioned co-relation. The gastric pH value, however, has been flattened by the averaging process in a way not doing justice to the range of reaction of the gastric juice as shown in the individual experiments.

SUMMARY OF SECTION 1.

The series of experiments presented would lead to the following conclusions:

1. An alkaline tide in the urine is the usual phenomenon after food in the normal individual on a mixed diet.

2. Inexplicable absences have been found in two cases.

3. Morning and afternoon tides of equal magnitude are by no means uncommon.

4. Either the morning or the afternoon tide may be the greater.

5. The "acid tide" is present, but lasts for about one hour only after a meal, when the alkaline tide develops.

6. After the evening meal a steady fall in pH is usual, until the pH reaches an equilibrium, and remains at a low level until the following day's breakfast is taken. Eating of certain foods during the night disturbs this equilibrium, giving a marked alkaline tide.

7. There is no reason to postulate lessened pulmonary ventilation, as suggested by Leathes, to explain the nocturnal acidity of the urine, for the evidence advanced in this paper strongly suggests that the acidity of the urine is a starvation effect.

8. An alkaline tide after supper is not usual, but may occur. In two cases where it did occur the gastric acidity was determined, and was found to show an acid phase corresponding to the alkaline phase of the urine.

Hence it was strongly felt that diet plays the major rôle in determining the reaction of the urine, and it was resolved to investigate certain diets in relation to the alkaline tide thereby produced.

SECTION 2. RELATION OF THE ALKALINE TIDE TO VARYING TYPES OF DIETS.

Howe and Hawk (17) have shown that, during extended fasting, the urine grows increasingly acid, till it reaches equilibrium at a pH of 5·63 (compare with night urine, Curves 3–5 of this paper). A protein diet was then given, and

the pH rose to 9·0 (apparently carbon dioxide was not conserved). But Torres (quoted by Robinson, Stearns, and Daniels, 29) found that protein increased the titratable acidity of the urine of infants, an observation not sustained by Robinson, Stearns, and Daniels, whose findings, however, did not show such an alkalinity as might be expected from the work of Howe and Hawk.

Lutz (11) describes an alkaline tide three hours after eating potatoes, meat, or greens; less so after eggs. This is followed by an acid tide. In fasting, the urine is acid. An alkaline tide was not found following oatmeal or bread-and-milk, or whilst fasting. Pesopulos finds a slight alkaline tide after fats or carbohydrates, a larger one after protein, and the largest on a mixed diet. Meyer (12) draws attention to the finding by Magnus Alsleben that the urine of meat-eating dogs is not acid, but alkaline. Meyer finds that a protein-rich diet in man gives a urine approaching to alkalinity, but rather strangely observes that a greater alkalinity could be produced by a protein-poor diet. Mixed diets lie intermediate. Very large alkaline tides were recorded on diets of casein and Witte's peptone. Noteworthy was the absence of the alkaline tide on a diet of olive oil, for olive oil is said to inhibit gastric acid-secretion, and the experiment further strengthens the view that the alkaline tide is due to gastric HCl-production. Meyer also observes that ingestion of large doses of glucose and laevulose produce no alkaline tide, though profoundly altering the gas-exchange.

Experimental.

(a) *Protein.* The present writer has found an abundant alkaline tide to be usual after meals rich in protein, e.g., eggs, soup, fish, meat, and meat-extractives. Supper is an exception to this, but may have a special explanation to account for it. In the following experiment a diet of meat and bread (somewhat loosely-controlled) was taken, and the urinary pH estimated hourly over 24 hours.

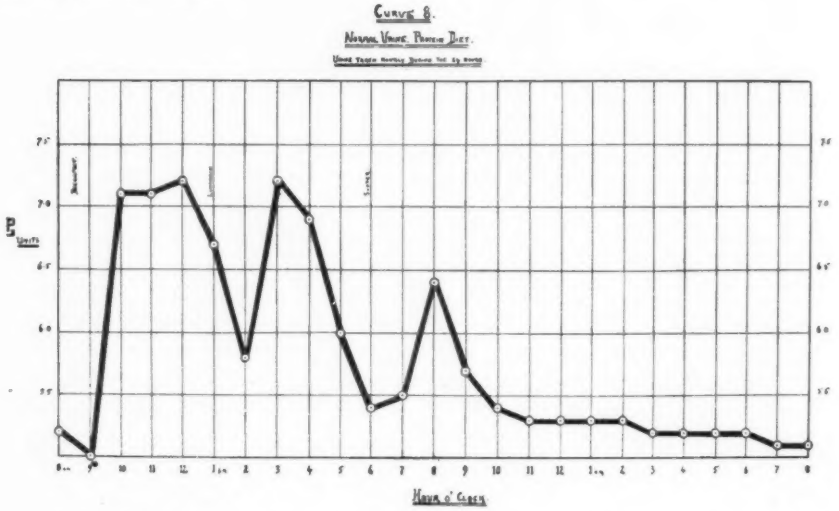
TABLE 5.

Normal Urine. High Protein Diet.

Normal Urine.		High Protein Diet.	
Hour.	pH.	Hour.	pH.
9 a.m.	7·2	10 p.m.	6·0
10 „	6·8	11 „	5·3
11 „	7·0	12 „	5·3
12 „	6·8	1 a.m.	5·1
1 p.m.	6·3	2 „	5·2
2 „	6·3	3 „	5·2
3 „	6·3	4 „	5·2
4 „	6·1	5 „	5·2
5 „	5·7	6 „	5·5
6 „	5·5	7 „	5·7
7 „	5·2	8 „	5·8
8 „	5·9	9 „	5·7
9 „	5·7		

This table shows a marked alkaline tide in the morning, with less than the usual 1 o'clock return to acidity. Instead of an alkaline tide, a plateau is seen

during the afternoon, with subsequent fall in the pH until after supper. An alkaline tide, its peak 4 hours after supper, obtains during the evening. Finally, the pH falls to its acid night level, from which it is disturbed by a meal at 4 a.m., with resulting alkaline phase. A more rigid diet was, however, desirable, and the writer passed 24 hours on the following regimen, approximately 12 ounces of lean cooked meat, and no other food, being taken at 8 a.m., 1 p.m., and 6 p.m. The hourly curve of urinary reaction is shown in Curve 8. Enormous morning and afternoon alkaline tides are seen. A brief alkalinity, almost complying with the definition of an alkaline tide, follows supper. Thereafter the urine becomes steadily acid, and, being undisturbed by food, remains so throughout the night.

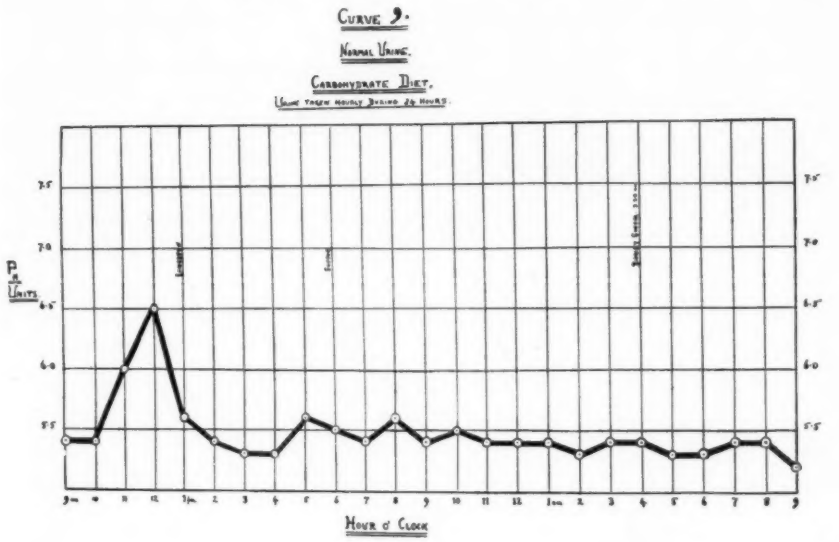


(b) *Carbohydrate.* The urinary reaction on carbohydrate diet is set out in Table 6 and Curve 9.

TABLE 6.

Normal Urine. Carbohydrate Diet.

Date.	Hour.	pH.	Hour.	pH.	Date.	Hour.	pH.	Hour.	pH.
18-	8 a.m.	5.4	9 p.m.	5.5	29-	9 a.m.	5.1	10 p.m.	5.5
19/5/28	9 „	5.6	10 „	5.5	30/11/28	10 „	5.2	11 „	5.3
	10 „	5.6	11 „	5.6		11 „	6.8	12 „	5.3
	11 „	5.3	12 „	5.6		12 „	7.0	1 a.m.	5.3
	12 „	6.0	1 a.m.	5.5		1 p.m.	6.0	2 „	5.2
	1 p.m.	5.3	2 „	5.3		2 „	5.5	3 „	5.4
	2 „	5.3	3 „	5.4		3 „	5.2	4 „	5.3
	3 „	5.5	4 „	5.5		4 „	5.3	5 „	5.3
	4 „	5.3	5 „	5.4		5 „	5.9	6 „	5.3
	5 „	5.3	6 „	5.4		6 „	5.6	7 „	5.5
	6 „	5.5	7 „	5.5		7 „	5.4	8 „	5.4
	7 „	5.4	8 „	5.3		8 „	5.5	9 „	5.2
	8 „	5.8				9 „	5.4		



Breakfast consisted of boiled rice, with a little milk. The other meals comprised "Uneeda" biscuits and approximately 100 grammes of barley-sugar. The experiment of 18/5/28 shows an acid urine throughout, with no alkaline tide. That of 29/11/28 shows an alkaline tide after the rice breakfast. This may have been due to psychical secretion of gastric HCl, due to the desire for food following a scanty and unpalatable meal. The barley-sugar meals, even though 80 grammes were taken at 3 a.m. in each case, were followed by no alkaline tide whatever.

Table 7 shows the hourly urinary reaction on a diet of cereal, toast, jam, and tea. Neither morning nor afternoon alkaline tides were present.

TABLE 7.

Normal Urine. Carbohydrate Diet.

Hour.	pH.	Hour.	pH.
8 a.m.	6.0	1 p.m.	5.6
9 „	6.0	2 „	5.4
10 „	6.4	3 „	5.6
11 „	6.2	4 „	5.8
12 „	6.0		

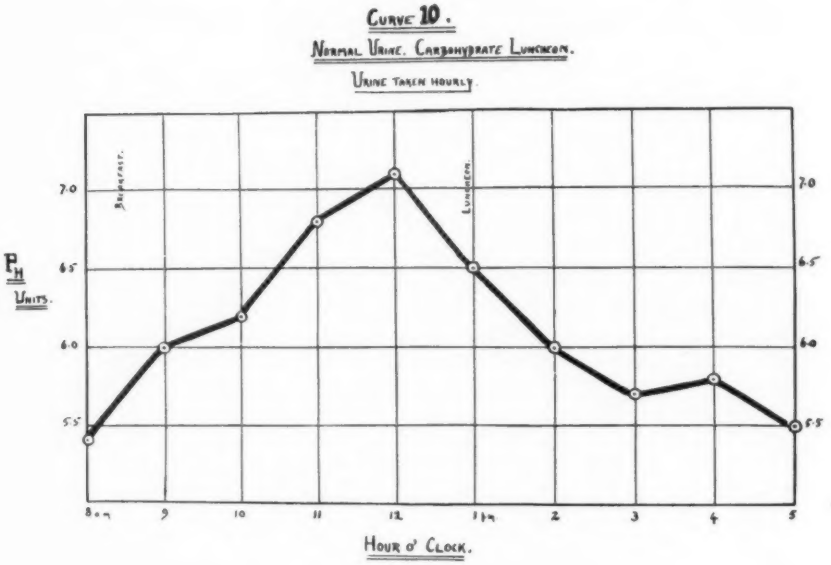
In Table 8 are the results of experiments where a luncheon of carbohydrate (bread, milk, honey) was taken, following on the usual breakfast rich in protein. The results are "averaged" in Curve 10.

TABLE 8.

pH. Normal Urine. Carbohydrate Luncheon.

Hour.	19/6/28.	3/7/28.	?	2/10/28.
8 a.m.	5.4			5.6
9 „	5.7	5.8		5.6
10 „	5.9	6.2	6.8	5.9
11 „	6.5	6.9	7.0	7.0
12 „	7.1	7.2	7.0	7.3
1 p.m.	6.0	6.8	6.9	7.0
2 „	5.6	6.6	6.4	6.1
3 „	5.4	6.0	5.7	5.5
4 „	5.3	5.9	5.9	6.0
5 „	5.3	5.9	6.2	5.5

Morning tides are well marked; the complete absence of afternoon tides is striking. Carbohydrate is, as Bolton (30) shows, a poor stimulant to gastric HCl-secretion, a fact to be remembered in interpreting these results.



(c) *Morning Fasting.* Leathes has described an alkaline tide in morning fasting, an observation confirmed by many others. Lutz (11), however, did not observe one, so further investigation seemed desirable. In the experiments detailed in Table 9, and reduced to a "mean" in Curve 11, breakfast was entirely omitted.

TABLE 9.

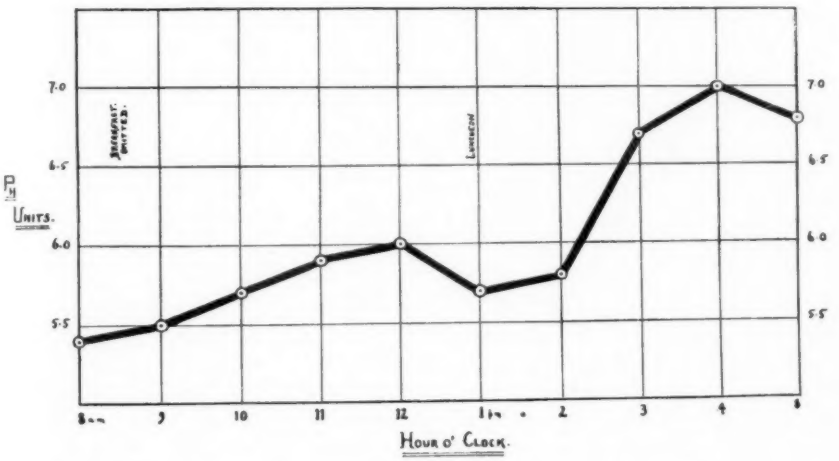
pH. Normal Urine. Morning Fasting.

Hour.	5/7/28.	6/7/28.	9/7/28.	8/7/28.	12/7/28.	13/7/28	?	?
8 a.m.	5·6	5·3	5·3	5·3	5·5	5·6		
9 „	5·3	5·2	5·3	5·3	5·4	5·9	6·0	5·7
10 „	5·6	5·2	5·2	5·2	5·6	7·0	6·2	5·5
11 „	5·6	5·2	5·7	5·3	5·9	7·3	6·0	6·0
12 noon	6·7	5·0	5·5	5·2	5·5	7·4	6·0	6·4
1 p.m.	6·2	5·0	5·5	5·3	5·4	7·1	6·0	5·4
2 „	5·9	5·0	5·9	5·3	5·7	6·6	6·1	5·7
3 „	6·9	6·2	7·2	7·5	6·5	7·2	6·2	5·7
4 „	7·1	7·0	7·3	7·0	6·9	7·5	6·4	6·9
5 „	6·9	6·7	6·9	6·0	6·9	7·3	6·4	7·1

CURVE 11.

NORMAL URINE.

MORNING FASTING.



One curve (undated) is of anomalous "flat" type (see Table 2). Of the rest, an alkaline tide occurs in three cases and is absent in four. Leathes' observation is thereby again confirmed, and it was to understand the meaning of this fasting tide that the following experiments were undertaken.

SECTION 3. ALKALINE TIDE AND GASTRIC FUNCTION.

Literature.

Observers have connected the alkaline tide with the gastric acid-secretion because: (a) the tide is usual 3 hours after meals; (b) it is well defined after

meals rich in protein and meat-extractives; (c) it is poorly defined after carbohydrate meals.

Munford and Hubbard (1, 31) state that the tide is absent in 80% of their cases of achlorhydria and very deficient acid-secretion. The depth and duration of the tide does not agree with the amount of acid or the form of the acid curve. On the other hand, Watson (9) finds a tide in the urine of three clinical achlorhydria cases. In several subjects development of alkalinity in the urine preceded gastric acid-secretion. A similar result occurred if the gastric secretion were delayed by administration of atropine. Watson therefore denies the correlation of gastric and urinary reaction, though his results might have been more valuable had they been obtained on diets stimulating to acid-secretion, such as peptone or meat-extract.

Schulten (32) has published work which so well agrees with the present writer's views that it seems necessary to state that Schulten's work appeared in Germany in May, 1928, and was not seen by the writer till some months subsequently. Schulten insists on a test meal exciting full gastric function, and uses one of meat, vegetables, etc., resembling the present writer's "mixed diet." He takes as his standard of achylia the response to this test meal, not to a carbohydrate one. In a wide series of achylia cases so defined, Schulten confirmed the findings of Munford and Hubbard that absent gastric acid-secretion is associated with absence of alkaline tide. In one patient an acidity of the stomach was induced by giving soup, when (and only when) an alkaline tide occurred in the urine. Schulten thus concludes that gastric secretion is the main (though not the only) cause of the alkaline tide.

Jansen and Karbaum suggest that reabsorption of hydrochloric acid from the gut produces the alkaline tide. Schulten shows that this cannot be so, since taking of acid by mouth gives no alkaline tide in achlorhydria patients. He suggests, however, that gastric HCl may be an excretion as well as a secretion (Katsch), and that the kidneys act vicariously in removing acid when the stomach (as in achylia) can no longer excrete; an untenable view if we accept the work of Scheer (33), who states that, on giving hydrochloric acid to infants, the urinary pH can be made to fluctuate widely, but that of the faeces remains almost unaltered.

Experimental.

Five subjects were employed. Four experiments (3/8/28, 4/8/28, 7/8/28, 8/8/28) were performed upon the present writer. The technique is tedious and volunteers scanty. Moreover, vomiting or derangement of stomach function are not unusual as the result of excessive swallowing of the stomach tube.

Methods. A Ryle's tube is passed every hour from 9 a.m. to 5 p.m. Samples of gastric content are withdrawn from different levels of the stomach (Kinsella, 34), and received into stoppered tubes to avoid loss of CO_2. The urine is collected as usual, hourly, from 9 a.m. to 5 p.m. To excite better secretion of

HCl than obtains with the usual carbohydrate test meal, the results whereof may be in this respect actually misleading to the clinician, a mixed diet is employed. Breakfast comprises two boiled eggs, porridge or cereal, toast, and tea; luncheon, soup, mincemeat, bread and butter, tea. No food is allowed between meals, and even water is discouraged.

The pH of the urine is obtained. The pH of the gastric content and its titratable acidity, in terms of cubic centimetres of N/10 NaOH per 100 cc. of gastric juice, are elicited. Titratable acidity, however, is but approximate, owing to the colour of the test meal, for which same reason estimations of free HCl were abandoned. The pH work is effected with the quinhydrone electrode, and is probably accurate to 0·05 pH unit.

Results. These are set out in Table 10 and "averaged" in Curve 12.

TABLE 10.

Normal Urine. Combined Gastric and Urinary pH Values.

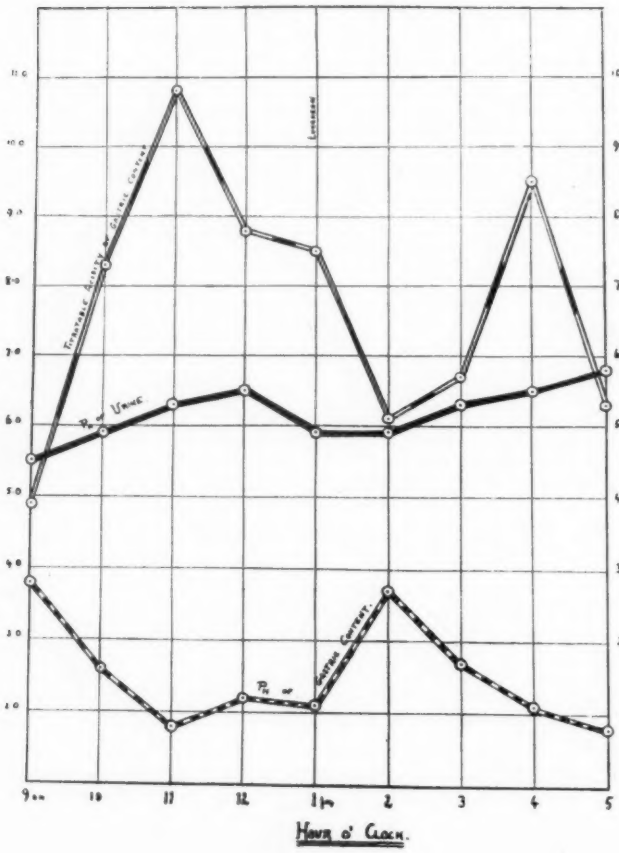
Date.	Hour.	pH Urine.	pH Gastric Content.	Titratable Acidity Gastric Content.
3/8/28	9 a.m.	5·4	4·3	
	10 „	5·4	3·6	35
	11 „	5·9	2·2	68
	12 noon	6·3	2·1	90
	1 p.m.	5·2	2·3	51
	2 „	5·5	3·9	26
	3 „	5·6	3·9	34
	4 „	6·1	2·2	79
	5 „	6·5	2·0	74
4/8/28	9 a.m.	5·8	4·0	37
	10 „	5·8	3·2	90
	11 „	6·7	1·8	158
	12 noon	7·1	2·2	87
	1 p.m.	6·3	2·2	70
	2 „	6·3	3·7	68
	3 „	7·0	2·7	45
	4 „	7·4	2·1	80
	5 „	7·1	1·8	41
7/8/28	9 a.m.	5·4	3·9	47
	10 „	6·9	2·4	91
	11 „	7·2	1·5	118
	12 noon	7·2	2·3	119
	1 p.m.	6·3	1·3	102
	2 „	6·3	4·6	47
	3 „	6·4	3·3	85
	4 „	6·7	2·6	90
	5 „	6·8	1·5	43
8/8/28	9 a.m.	6·0	3·6	66
	10 „	5·7	2·4	87
	11 „	7·0	1·6	110
	12 noon	7·3	1·3	103
	1 p.m.	7·0	1·5	102
	2 „	6·6	4·0	71
	3 „	7·3	2·5	—
	4 „	6·8	2·2	100
8/10/28	9 a.m	5·2	4·4	15
	10 „	—	2·0	68
	11 „	5·2	2·0	43
	12 noon	5·2	3·1	16
	1 p.m.	5·2	2·1	30

Date.	Hour.	pH Urine.	pH Gastric Content.	Titratable Acidity Gastric Content.
	2 p.m.	5.0	4.0	28
	3 „	5.5	2.1	50
		(Subject declined to proceed.)		
18/10/28	9 a.m.	5.5	2.8	33
	10 „	5.9	1.9	66
	11 „	5.9	1.9	90
	12 noon	5.7	2.0	56
	1 p.m.	5.4	3.1	96
	2 „	5.5	2.2	66
	3 „	5.8	1.9	70
	4 „	5.6	1.4	78

CURVE 12.

NORMAL URINE. MIXED DIET.

COMBINED CURVE OF URINE & GASTRIC CONTENT.



Curve 12, though the range of urinary reaction has been unduly flattened by the averaging process, is to be taken in conjunction with the two experiments of Table 4 and Curve 7. The normal variations of gastric acidity are well defined, lying between pH 4·6 and pH 1·3, with maximal acidity some 3 hours after meals, i.e., 11–12 a.m. and 4–5 p.m. If gastric acidity be the cause of the alkaline tide of urine, the peak alkalinity of the urine should fall within these hours. This is seen to be not mathematically so, yet the divergencies are not sufficient to disable the inter-relation theory. In general, an alkaline phase is manifest in the urine during the time a phase of increased acidity appertains in the stomach contents. Individual variations are very likely, in that mucus secretion and possible duodenal regurgitation will cause a reduction of the H ion concentration of the gastric content not paralleled in the urinary reaction. The general relationship of the acid phase in the stomach content with an alkaline phase in the urine is held to be very striking and suggestive. The experiences of other workers are awaited with interest.

The curve for 8/10/28 presents a very complete vagary, in that there is normal gastric acidity, a very low titratable acidity, and no alkaline tide at all. The patient, however, had undergone appendicectomy nine days before, and might be better left out of the series, as we do not know what uncharted reflexes such an operation may set up.

The titratable acidity is much greater than the "normal" values of clinical test meal work, for which the nature of the diet accounts. It is also very variable in its relation to the pH of the gastric contents, presumably from the buffering effect of the diet, and from the influence of dilution, which alters titratable acidity far more extensively than pH values.

Discussion. It is held by Dodds, after Bennett and Ryle, that in seeking an alkaline tide, achlorhydria should be first excluded (4). Munford and Hubbard (31) have elevated the alkaline tide into the status of a test, absence thereof giving presumption of achlorhydria. Criticized by Watson (9), their views are supported by Schulten (32). The present writer would insist on a better criterion of achylia than the clinical test meal at present in vogue.

Boyd and Austin (35) find that in Pavlov-pouch dogs an alkaline tide is no more usual than an acid tide in the urine, and they do not accept the inter-relation view. But their dogs were fed upon bread and milk, an unnatural diet for a carnivore, and one exciting little HCl-secretion in omnivorous man. Nor is the Pavlov-pouch free of all criticism as a mirror of gastric activity.

The writer believes that efficient team-work is called for both upon the abnormal stomach of Schulten's researches and upon the problems in the normal suggested in the present paper. Normal diets, morning fasting, and special diets all require investigation by the combined gastric and urinary technique. It would thus be possible to set out in parallel the range of gastric and urinary pH, the pH of the blood, the CO_2 exchange, the NH_3 nitrogen of the urine, a

procedure likely to solve the problem in a way impossible to the individual worker. Above all, it is thought that illuminating information will lie in the study of the pH of the blood.

SECTION 4. DRUGS AND URINARY REACTION.

(a) *Acidification of Normal Urine.*

Henderson and Palmer (22) find that urine, poorly buffered against changes towards alkalinity, is heavily buffered against changes in the direction of acidity. The acid and basic phosphates and acid and basic carbonates (Gamble, 7) are the buffer salts. Indeed, Henderson finds that ingestion of 10 grammes of NaH_2PO_4 produces a urinary pH not lower than 4·70. This value he advances, and Marshall (20) accepts, as the probable lower limit of pH in the urine of healthy man. The writer, using massive doses of HCl, has seen no urine more acid than pH 4·8.

Hydrochloric Acid. It is shown by Maclean and Griffiths (36) that its administration immediately after a meal renders unnecessary the secretion of gastric HCl. Hence sufficient doses of acid should abolish the alkaline tide in urine, should that tide be caused by gastric acid-secretion.

Meyer (12) succeeded in abolishing the alkaline tide in two experiments by administering 5 cc. of 2·5% HCl. In another series he found that casein + HCl gave a greater alkaline tide than casein alone, a result wholly opposed to his first findings and to the inter-relation theory. However, it is possible that his dosage of acid was insufficient to abolish gastric secretion, since even a dog on sham-feeding produces 124 cc. of 0·6% HCl, equivalent to nearly 26 cc. of 2·5% (Pavlov, 37). It was decided to employ much larger doses of HCl, and in the first experiments employed 1 drachm of 10% HCl, or approximately three times Meyer's dosage. The acid was flavoured with a drachm of Syr. Limonis B.P., at 9 a.m. and 1 p.m., after "mixed" meals. The results are presented in Table 11.

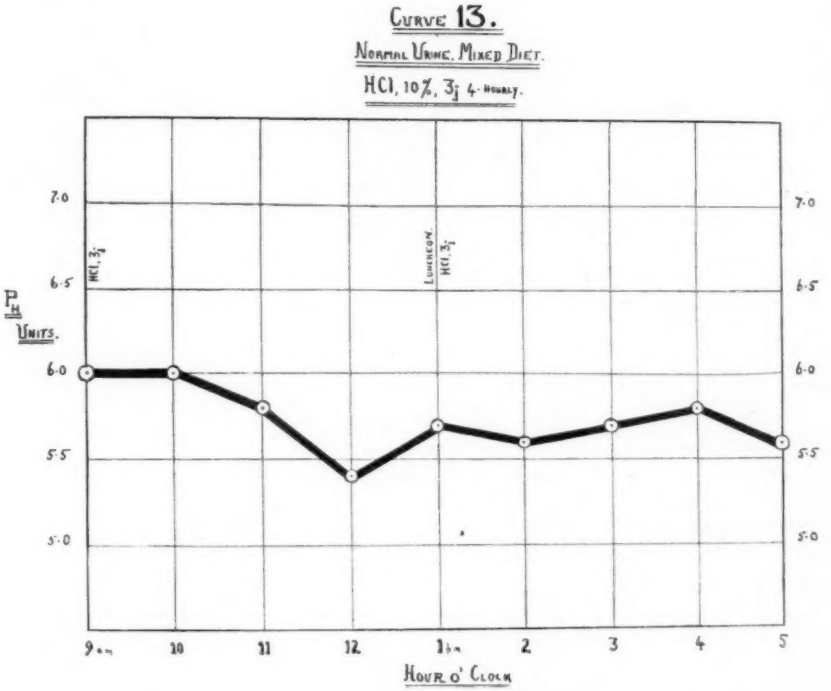
TABLE 11.

Normal Urine. Mixed Diet. HCl 10%, 3j at 9 a.m. and 1 p.m.

	pH.			
Hour.	7/1/29.	7/1/29.	8/1/29.	14/1/29.
9 a.m.		6·8	6·8	5·4
10 „	6·0	6·3	5·5	5·7
11 „	5·9	5·6	5·7	5·5
12 noon	6·0	6·0	5·5	5·4
1 p.m.	6·7	5·7	5·7	5·4
2 „	6·2	6·1	5·3	5·5
3 „	6·1	5·1	5·7	6·2
4 „	6·5	5·2	6·2	5·7
5 „	5·7	5·9	5·6	5·7

In no case is an alkalinity seen, conforming to the definition of an alkaline tide. Attention is drawn to the high initial pH values in two of the curves,

both from one subject. They are much higher than the "typical" values given in Table 1. In one case at least the breakfast contained meat, which may explain the high initial pH reading (see Curve 8). In another series of experiments the same dose of acid, unflavoured, produced parallel results, i.e., an alkaline tide in but one case. The maximal B.P. dose of NaH_2PO_4 produces results substantially identical with those due to HCl administration.



Hydrochloric acid, then, taken by mouth in sufficient doses, generally abolishes any definite alkaline tide. But minor fluctuations are not unusual, and it was thought to attempt abolition of these by pushing the acid to the limit of tolerance. The acid, accordingly, flavoured with an equal amount of Syr. Limonis B.P., was given as under to subjects on "mixed" diet. At 9 a.m., after breakfast, ½ drachm is taken; at 10 a.m., 1 drachm; at 11 a.m., ½ drachm. Larger doses were not tolerated, and for this reason the taking of acid terminated during the forenoon, though pH readings were taken throughout the day. The results are expressed in Table 12.

TABLE 12.

Normal Urine. Mixed Diet. HCl 10%, 3ij between 9 a.m. and 11 a.m.

Hour.	10/2/29.	?	pH. 19/2/29.	20/2/29.	20/2/29.
9 a.m.	5.2	5.3	5.7	6.4	5.3
10 „	5.2	6.9	5.4	6.2	5.2
11 „	4.9	6.7	5.3	6.1	5.3
12 noon	5.0	6.5	5.4	5.3	5.0
1 p.m.	4.8	5.9	5.1	5.0	5.0
2 „	4.9	5.6	4.9	4.9	5.0
3 „	5.0	6.0		5.0	5.1
4 „	5.0	6.0	5.0	5.0	5.0
5 „	5.0	5.9	5.0	5.0	5.1

In the first experiment of 20/2/29 breakfast had been taken one half-hour earlier than in the rest of the series. Of five experiments, the alkaline tide is abolished in four. This is regarded, when taken together with Table 11, as being strong evidence in favour of the view of the gastric origin of the alkaline tide.

SUMMARY.

1. The literature of the alkaline tide in particular, and of the reaction of the urine in general, has been critically surveyed.

2. The alkaline tide in urine has been shown to be usual on a mixed diet. It usually reaches a peak some 3–4 hours after the taking of food, and is often preceded by a brief phase of acidity, which last is found to coincide with a phase of low gastric acidity.

3. The alkaline tide has been found to be completely absent in three experiments upon normal individuals.

4. Evidence has been presented that protein excites a marked alkaline tide, whilst carbohydrate is associated with an acid urine.

5. The explanation advanced by some observers, that the alkaline tide is primarily due to respiration, is shown to be untenable, though a subordinate influence is not denied.

6. Combined curves of the reaction of the gastric content and of the urine have been made in a small series of cases, indicating that the alkaline phase of the urine is accompanied by a phase of increased acidity of the stomach contents, and *vice versa*. This co-relation is not mathematically exact, and must be influenced by such factors as temperature, respiration, mucus secretion, and possible duodenal regurgitation, yet it is sufficiently strong as to suggest gastric acidity as the dominant factor in the causation of the alkaline tide in urine.

7. Absence of an alkaline tide after the evening meal is usual, but not invariable. Two cases, which have shown an alkaline tide after the evening meal, have shown simultaneously an acid tide in the stomach contents.

8. The nocturnal urine in the waking subject tends to reach an equilibrium

some 5 hours after the evening meal. It is of an acidity of the order of 5·3 pH, and tends to remain uninterruptedly so throughout the night, unless disturbed by the taking of food.

9. The alkaline tide is usually abolished by taking the maximum B.P. dose of sodium hydrogen phosphate.

10. The administration of sufficient doses of hydrochloric acid tends, in the great majority of cases, to the abolition of the alkaline tide. This is presented as good evidence for the view that gastric acid-secretion is the dominant cause in the production of the alkaline tide of urine.

SECTION 5. CLINICAL APPLICATIONS.

1. *The Alkaline Tide as a Test in Acidosis.*

Palmer, Salveson, and Jackson (38) suggest that, since the urinary pH does not reach 7·4 until the plasma-bicarbonate CO_2 exceeds the 71 ± 5 vols. % of Palmer and Van Slyke, the reaction of the urine is a valuable guide to acidosis, especially in cardio-renal disease. Hence sodium bicarbonate should be administered till the urinary pH exceeds 7·4, short of which the acidosis is uncontrolled. It must be rare that facilities for direct estimation of alkali reserve are wanting in the presence of facilities for pH estimation, but the method may have clinical value should universal indicators, such as Bogen (39) describes, enter general clinical use.

2. *Alkaline Tide and Alchlorhydria.*

The writer has tested Munford and Hubbard's views on but one clinical achlorhydric, who admittedly did show an atypical, but definite, alkaline tide. Further investigation seemed unwise till some more efficient test meal should have been devised, a view confirmed by Schulten's findings. Should the work of Munford and Hubbard stand the ultimate proof, a valuable test of gastric acidity will exist for use in cases where the Ryle's or Rehfuss's tube cannot be swallowed. The finding of a satisfactory universal indicator, accurate to 0·2 pH unit between 5·5 and 8·5 pH, would bring such a test into general clinical application.

3. *Alteration of Urinary Reaction.*

It is suggested that carbohydrate diet may have value in promoting an acid urine, and that hydrochloric acid (10%, flavoured with Syr. Limonis B.P., 1 drachm 4-hourly, well diluted) may succeed in cases where sodium acid phosphate fails. Where alkalinization of the urine is desired, a protein diet, if not contraindicated on general clinical grounds, may have value.

A study of the urinary reaction in pyelitis will be a continuation of the present work.

ACKNOWLEDGMENT.

The writer desires to convey his thanks to the Trustees and to Dr. Penfold of the Baker Medical Research Institute for the facilities made available to him.

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THE ROLE OF ENZYME ACTION IN THE FORMATION OF DENTAL CALCULI[1]

by

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Previous Work on Dental Calculus.

The occurrence of calculus, or so-called tartar, in and around the mouth is looked upon by the dental profession generally as an unavoidable evil. It is universally recognized that the gingival tissues as a rule are much healthier when the teeth are free from such deposit, but the exact nature of the calculus and its aetiology have not received the amount of consideration they merit.

Several theories have been formulated to explain these deposits. Burchard (1895) considered them as due to the action of lactic acid, formed in the mouth, upon the secretion of the salivary glands, thus producing a coagulum of mucin, in which deposits of lime salts (mainly what he calls lacto-phosphates) become entangled.

It has also been attributed to the precipitation of calcium salts from the saliva by the loss of the CO_2 which held them in solution. This precipitation would be facilitated by the presence of ammonia, which is stated to be present in some cases, from the decomposition of protein substances in the mouth. Such salts as calcium carbonate and tricalcium phosphate would act in this way and be deposited, in association with mucin, as calculus or tartar. Kirk (1911), Talbot (1913), Kelsey (1914).

Black (1915) considers tartar as a mixture of calcium phosphate and smaller amounts of calcium carbonate, held together in a mass by an organic compound, which, in his opinion, is formed after these salts have been deposited upon the teeth. This mass, which he refers to as calco-globulin, finally becomes hardened by the decomposition of much of the colloidal matter.

Prinz (1921) held that the deposition of tartar in the mouth was the result of more than one process. The primary factor, in his opinion, was the concentration of colloids upon the surface of saliva in conformity with Gibb's law of

[1]This work was carried out during the tenure of a Fred Knight Research Scholarship of the University of Melbourne, and formed part of a thesis for the Degree of Doctor of Dental Science.

surface tension; then the solvent power of the saliva was physically diminished, resulting in the liberation of crystalline calcium salts, which at once became caught in the colloidal stroma. This physical action might also be enhanced by the chemical reactions discussed above.

In his experimental work Prinz attempted to produce tartar deposits from saliva, and also from a synthetic saliva. He accomplished the first by placing a series of teeth partially embedded in modelling compound in the incubator at 40°C., and filling the artificial pockets daily with fresh saliva. Within eight or ten weeks incrustations formed on the roots, which appeared in every respect to be identical with the tartar found in the human mouth. To prepare artificial saliva Hammerbacher's (1881) analysis of saliva ash was taken as a basis, the colloidal menstrum being obtained by dissolving 2½ grains of dried powdered albumen in 1,000 parts of distilled water. Using the artificial saliva instead of the normal saliva, he repeated his first experiment, and obtained deposits on the teeth which were from all outward appearances similar to those obtained from the natural saliva.

Bulleid (1925) noticed that wherever tartar deposits were found round the teeth *Leptothrix Buccalis* filaments were always present. He therefore suggested that these organisms might play some part in the deposition of calcarious deposits around the mouth. He performed both qualitative and quantitative experiments, using 1 in 1,000 calcium chloride solution as the medium, and adding to this nutrient broth. Tubes containing the mixture were inoculated with the Leptothrix and various other mouth organisms and incubated for four days. Growth was obtained in all tubes except a control, but only those innoculated with the Leptothrix showed any deposit of calcium salts. In addition he performed experiments on cats, which gave very inconclusive results. The animals were anaesthetized, the jaws propped open, and the gum all round the teeth in both jaws was reflected on the external aspect to the depth of a quarter of an inch. In one animal used as a control an incision only was made; in the other a small amount of a culture of Leptothrix was inserted. Tartar appeared on the teeth in both animals, but the period of time in the control was longer than in the infected animal.

The Composition of Dental Calculus.

Many investigators have made analyses of dental calculus, and though their actual results vary a great deal, it has been shown to consist of about 22% water and organic matter (the portion removable by burning the calculus) and about 78% inorganic matter (the proportion removable by decalcification with acids). All these observers are practically agreed upon the substances present in calculus as mainly calcium phosphate, with some calcium carbonate, calcium fluoride, and magnesium phosphate combined with organic matter.

There are great variations in the analyses of saliva which have been quoted in the literature. This is due possibly to the difficulty in isolating the organic constituents, and to the fact that mixed saliva varies in quality and quantity with the nature of the food taken into the mouth.

Most of these analyses fail in one respect, namely, the exact nature of the organic material which is known to be present. The reason for this probably lies in the method adopted in the majority of cases, where as a rule the percentage of organic matter has been determined by ashing.

Two other substances not mentioned in these analyses have since been shown to be present in calculus. The first of these is calcium oxalate, which was first demonstrated by Badanes (1928). The other is cholesterol, which the present author has recently demonstrated in a mixed sample of tartar collected from the mouth. As far as it is known this is the first time cholesterol has been shown to be present in tartar. A known weight of powdered tartar was taken and extracted with ether in a filter-paper thimble placed in a soxhlet extractor. The ether was then evaporated, and the residue dried and weighed. It was found to be about 3% of the total weight. This residue was shown to contain cholesterol.

The Formation of Calcium Phosphate Deposits by Enzyme Action.

Within the last few years a new light has been thrown on the process of ossification of bone by Robert Robison (1923). He showed that the bones and ossifying cartilage of young animals contained an enzyme which rapidly hydrolyses hexosephosphoric esters, and glycerophosphoric esters with the liberation of free phosphoric ions. Liver, spleen, and pancreas were much less active in this respect, while muscle and non-ossifying cartilage were almost entirely without action on these phosphoric esters. A hexosediphosphatase, which splits off at least one of the phosphoric acid groups of the hexosediphosphoric ester, appeared to be more commonly distributed, being found in each of the tissues named above. Robison therefore suggested that "the former of these enzymes, i.e., the monophosphoric esterase present in bone, is concerned in the process of ossification, by effecting the hydrolysis of phosphoric esters contained in the blood, thus bringing about a local increase in the concentration of inorganic phosphate ions, so that the product of the concentration of phosphate and calcium ions becomes greater than the solubility product of calcium phosphate, which is therefore deposited in a solid state."

Robison (1924) also showed that such enzymes had a wider distribution than was first realized, being found in the bones of all animals so far examined, i.e., rat, rabbit, guinea-pig, kitten, chicken. They were also found in the wall of the intestine, and extracts prepared from the teeth of young and adult guinea-pigs, rats, and rabbits contained the enzyme in an even higher degree than that prepared from the bones of the same animals.

As it had also been proved to be present in the greater part of the alimentary canal, it seemed reasonable to suppose that an enzyme of a similar nature might be present in soft tissues of the mouth. If this proved to be the case it is also quite logical to suppose that, as blood serum contains a combined phosphate which can be hydrolyzed by this enzyme, so also might the saliva. Assuming for the moment that this were so, and that there were present in the tissues of the mouth an enzyme, if not identical, of a similar nature to that found in the other parts of the body previously mentioned, the result would be an increase of PO_4 ions in the saliva. In the words of Robison, "If a condition of equilibrium exists between solid calcium phosphate on the one hand and a solution containing un-ionized calcium and calcium and phosphate ions on the other, any increase in the concentration of either of these ions will result in further deposition of solid calcium phosphate."

Calcium phosphate is known to form about 65% of the inorganic salts which go to make up the hard deposits occurring in the mouth, therefore it is not unreasonable to suppose that such enzyme action is a means whereby tartar can be deposited in the mouth.

The fact that combined phosphoric acid occurs in the blood plasma has been established by many investigators, who have shown that blood contains, in addition to lipoid phosphorus and inorganic phosphate, a certain amount of combined phosphoric acid, which is readily hydrolysed by boiling with acids. Thus Greenwald (1916) found a small amount of such combined phosphoric acid in serum. Bloor (1918) found similar amounts of organic phosphate (10% of the total phosphates) in the plasma of normal men and women, but a much greater amount (60% to 80%) in the corpuscles.

The work of Kay (1928) on the hydrolysis of sodium and potassium pyrophosphate to the ortho-phosphate by enzyme action suggested another method whereby free phosphates might be liberated. Kay found that pyro-phosphatase was widely distributed in mammalian tissues, and that its distribution was similar to that of the ortho-phosphoric esterase previously described; the zone of optimal activity, however, was between pH 7·2 and 7·8, as against pH 8·8–9·3 for the latter enzyme. It was thought that possibly a similar action might play some part in the formation of dental calculi. Up to the present little work has been done along these lines.

The following experiments were carried out to ascertain whether an enzyme was present in the gingival tissues which would hydrolyse a phosphoric ester, whether the saliva contained combined phosphoric acid which could be hydrolysed by means of such an enzyme with the formation of free phosphates; and finally, as it has already been shown that blood plasma and whole blood do contain such combined phosphoric acid, whether the enzyme would react on them in a similar manner, producing a similar result.

EXPERIMENTAL.

Methods.

Throughout the following experiments two methods were used for the estimation of phosphates.

Method No. 1 was used for estimating the free phosphate present in solutions, and is a modification of Neumann's method, omitting the previous combustion with nitric and sulphuric acid.

The exact method employed was as follows: A solution containing 5 cc. of concentrated sulphuric acid, 25 cc. of a 50% solution of ammonium nitrate, and 60 cc. water was heated to 75–80°C. The phosphate solution to be estimated was then added, followed immediately by 20 cc. of a 10% solution of ammonium molybdate. If the volume of the phosphate solution was much greater than 10 cc. the amount of water was correspondingly reduced. The flask was then vigorously shaken, and allowed to stand for a quarter of an hour to half an hour. The yellow precipitate was then filtered off through a Gooch crucible, washed and estimated in the usual way by titration with standard soda solution, 0·1 N. being employed when the amount of free phosphoric acid corresponded to less than 0·2 mg. P. It cannot, of course, be taken for granted that no organic ester of phosphoric acid will be hydrolyzed under the above conditions, but actual tests showed that with solutions of glycerophosphoric acid the amount of hydrolysis was negligible, provided the temperature was not allowed to rise or to be prolonged unduly.

Method No. 2 was used for the estimation of combined phosphates after the free phosphates had been removed from the solution by magnesium citrate mixture.

Ten cc. of magnesium citrate were added to each tube, together with 5 cc. of 30% ammonia. This was allowed to stand for 24 hours, filtered, and the filter papers washed well with 30% ammonia solution to make sure that all the filtrate passed into the flask. The unprecipitated combined phosphate was estimated in this filtrate by the ordinary Neumann's method. In some experiments Brigg's method (Cole, 1926) for estimation of free phosphate was used.

Method of Obtaining Enzyme Extract. The technique was similar to that use by Robison.

The tissues were taken and thoroughly ground in a mortar with ten times their weight of water saturated with chloroform. They were allowed to remain at 0° during 24 hours, and then filtered. With the addition of a little chloroform, solutions prepared in this manner keep in the ice-chest for one to two months without any deterioration of their hydrolytic power. In all experiments the pH was adjusted to 9, and all incubations carried out at 37°C. for 48 hours. The results are expressed as phosphorus in milligrams.

Experiment No. 1.

On account of the difficulty of obtaining very much extract from human gum tissue an experiment was made using only 0·2 cc. of an extract of guinea-pig femur to ascertain whether experiments were possible with so small an amount. The glycerophosphate solution used was calcium glycerophosphate of a strength $\frac{M}{1000}$ The mixtures were set up as follows:

A. 0·2 cc. extract + 20 cc. glycerophos. gave 0·44 mg. P as free phosphate.
B. 0·2 cc. extract + 20 cc. water gave 0·22 mg. P as free phosphate.
C. 0·2 cc. water + 20 cc. glycerophos. gave nil as free phosphate.

Thus 0·22 mg. P has been liberated as free phosphate in A. This result showed that the experiment could be carried out quite satisfactorily with small quantities of enzyme.

Experiment No. 2.

This was carried out to find if the human gum tissues contained an enzyme of a similar nature to that described by Robison. The patient from which the gum was excised was a man of about forty-five. The mouth was reasonably clean, but had a fairly heavy and general tartar deposit, with a certain amount of marginal gingivitis, alveolar recession, and a pocket formation. The gums, however, were of a fairly normal colour, and did not have the bluish or cyanosed appearance which so often accompanies the condition described above. A gum resection was performed in the usual manner, and the soft tissues were washed well in normal saline, and then several times in distilled water. An extract was made as described above, and the mixtures were set up in duplicate as follows:

A. 20 cc. of glycerophos. + 0·2 cc. extract gave 0·325 mg. P as free phosphate.
B. 20 cc. water + 0·2 cc. extract gave 0·185 mg. P as free phosphate.
C. 20 cc. glycerophos. + 0·2 cc. water gave nil.

The first set was estimated by method No. 1, and showed that 0·140 mg. P was liberated as free phosphate in A.

To act as a check on this result the duplicates were estimated by method No. 2 by estimating the unhydrolyzed phosphate in the mixture after incubation, and any hydrolysis in A would be shown by the combined phosphate being less than the sum of the combined phosphate in B and C. The results were as follows:

A. 0·826 mg. P.
B. 0·209 mg. P.
C. 0·748 mg. P.

Phosphate hydrolysed = (B + C) — A = 0·131 mg. P.

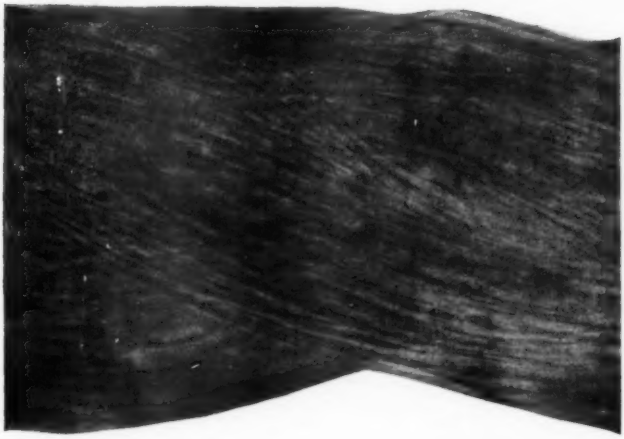

Fig. 1. Silk thread, no enzyme. Note the absence of calculus. Taken from tube No. 2 after 7 days.

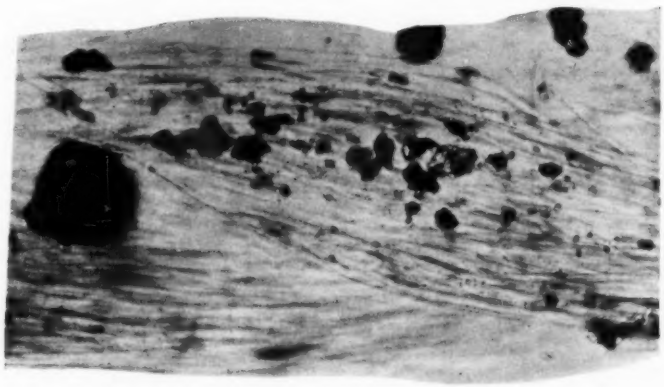

Fig. 2. Silk thread, with enzyme. Note the calculus in various stages of formation. Taken from tube No. 4 after 7 days.

PLATE 1.

Fig. 3. Another section of the same thread. Note the calculus.

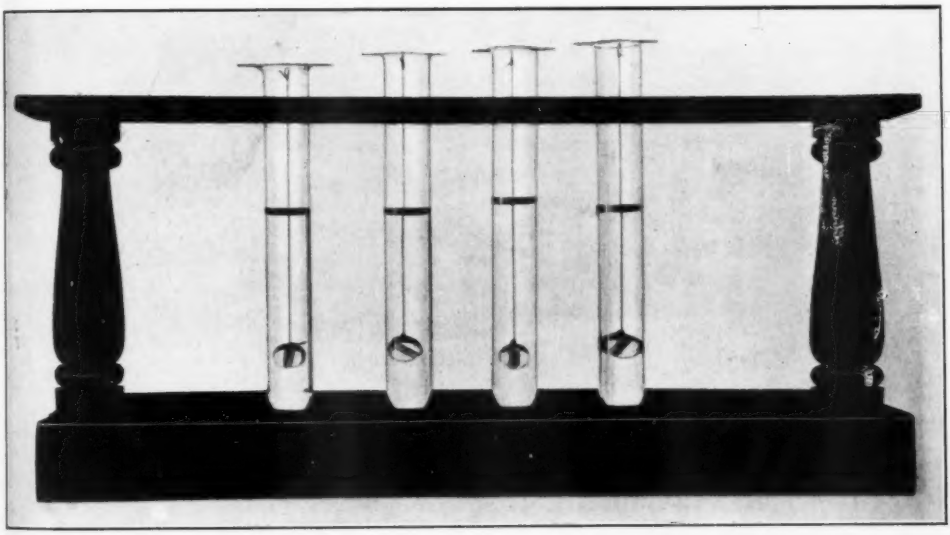

Fig. 4. Illustrating the apparatus used in Experiment No. 9.

PLATE 2.

Allowing for experimental error, the result is practically the same as obtained by the first method of estimation, thus furnishing adequate proof of the existence of the enzyme in the gingival tissues.

Experiment No. 3.

To make certain that this action was due to an enzyme a similar experiment was set up; the enzyme extract (from human gum tissue) used, however, was previously brought to the boil.

A. 20 cc. of glycerophos. + 20 cc. of extract gave 0·275 mg. P as free phosphate.
B. 20 cc. water + 2 cc. of extract gave 0·232 mg. P as free phosphate.
C. 20 cc. glycerophos. + 2 cc. water gave nil.

The free phosphate was estimated by method No. 1. Thus only 0·043 gms. of phosphorus (pract. nil.) was set free in A.

This result shows that boiling destroys the hydrolytic power of the extract; it is therefore safe to assume that the action was due to an enzyme. Kay found that it was necessary to boil for ten minutes before the action of enzymes of a similar nature in other parts of the body was totally destroyed.

Experiment No. 4.

Similar to Experiment No. 2. The tissue was removed from a woman aged 38, with a moderate deposit of calculus in the mouth. The same procedure was adopted, and an extract made from the tissue obtained. The experiment was carried out in duplicate, and the results estimated by method No. 2.

A. 20 cc. of glycerophos. + 0·2 cc. of extract gave 0·20735 mg. P and 0·20738 as combined phosphate.
B. 20 cc. of aqua dest. + 0·2 cc. extract gave 0·132 mg. P and 0·1738 mg. P as combined phos.
C. 20 cc. glycerophos. + 0·2 cc. aqua dest. gave 0·2271 mg. P and 0·22145 mg. P.
(B + C) — A = 0·1518 mg. P phosphate liberated in A and 0·1878 mg. P in A1.

From these figures it can be seen that there is a fairly large variation between the two sets of results. In spite of this both show a definite hydrolysis. These results, coupled with Experiment No. 2, seem to the author to give sufficient evidence of the presence of the enzyme.

Experiment No. 5.

To determine whether free phosphate could be formed from the saliva by enzyme action, using extract from a guinea-pig femur, the following mixtures were set up in triplicate, estimated by method No. 2.

A. 2 cc. saliva + 0·2 cc. extract gave 0·2541 mg. P and 0·2860 mg. P and 0·2354 mg. P.

B. 2 cc. water + 0·2 cc. extract gave 0·1903 mg. P and 0·1790 mg. P and 0·2068 mg. P.

C. 2 cc. saliva + 0·2 cc. water gave 0·2354 mg. P and 0·2530 mg. P and 0·2574 mg. P.

Thus 0·1716 mg. P phosphate were liberated in A, 0.1460 mg. P in A1, and 0·2290 mg. P in A2.

Although there is some variation between these results, it is seen that in every case a hydrolysis has taken place, and there has been a marked increase of free phosphate in the tube containing the enzyme, indicated, of course, in this experiment by a decrease in the combined phosphate as shown above.

These results would agree with the formation of free phosphate by enzyme action on some phosphate containing complex, possibly of the nature of a phosphoric acid ester.

Experiment No. 6.

To determine whether the extract from human gingival tissue, interacting with the saliva, would liberate free phosphate.

The subject was a woman aged 35, with a moderate deposition of tartar. The saliva was collected first, a gum resection was then performed, and the extract prepared as before. Tubes were set up as follows (method No. 2):

A. 4 cc. of saliva + 0·2 cc. extract gave 0·9405 mg. P and 1·0283 mg. P.

B. 4 cc. water + 0·2 cc. extract gave 0·9350 mg. P and 0·7106 mg. P.

C. 4 cc. saliva + 0·2 cc. water gave 0·9734 mg. P and 1·1770 mg. P.

Thus 0·9679 mg. P were liberated in A and 0·8591 mg. in A1.

These results point to the fact that human gingival tissues contain an enzyme of a similar nature to that described by Robison, which will hydrolyze a phosphate containing complex present in the saliva with a resultant formation of free phosphate.

Experiment No. 7.

As dogs are greatly subject to tartar deposits, it was decided to test the oral epithelium of the dog for the presence of this enzyme. A healthy animal was taken, killed, the oral tissue immediately removed, and an extract made as previously described. The kidneys and ureter were also removed, and extracts made from these, which were kept and used in later experiments. Mixtures were set up as follows, and the results estimated by method No. 2:

A. 0·2 cc. extract + 20 cc. glycerophos. gave 0·4334 mg. P and 0·4323 mg. P.

B. 0·2 cc. extract + 2 cc. of aqua dest. gave 0·3982 mg. P and 0·4037 mg. P.

C. 0·2 cc. aqua dest. + 20 cc. of glycerophos. gave 0·4178 mg. P and 0·5157 mg. P.

Thus 0·3826 mg. P liberated in A and 0·4873 mg. P in A1.

These results show clearly that such an enzyme is present in the oral tissue of a dog.

Experiment No. 8.

It has been previously shown by Robison that blood serum and whole blood contain a phosphoric ester which can be hydrolyzed by the enzyme contained in bone extracts.

The following experiment was carried out to determine whether the enzyme found in the oral tissues would give a similar result.

A. 0·2 cc. extract + 2 cc. blood serum gave 0·4367 mg. P and 0·4092 mg. P.

B. 0·2 cc. extract + 2 cc. aqua dest. gave 0·0814 mg. P and 0·0836 mg. P.

C. 0·2 cc. aqua dest. + 2 cc. blood serum gave 0·4620 mg. P and 0·4620 mg. P.

The results were estimated by method No. 2. Some red corpuscles were mixed with the serum. Thus 0·1067 mg. P were liberated in A and 0·1364 mg. P in A1.

These results agree sufficiently to show that the enzyme present in the gingival tissues will also form free phosphate from blood serum.

Experiment No. 9.

Having shown that human gum tissues contained the enzyme, and that there were present in the saliva combined phosphate which was capable of being hydrolyzed, and free phosphate formed by the action of this enzyme, it was thought that by imitating as nearly as possible the condition existing in the oral cavity, it might be possible to produce tartar *in vitro*.

For this experiment four small test tubes were taken and 5 cc. of saliva placed in each, and the pH adjusted to 9. Tubes 1 and 2 were left just as they were, but 0·2 cc. of extract were added to 3 and 4. Extract from a dog's mouth was used instead of the human, as extreme difficulty was experienced in obtaining cases from which the necessary tissue could be removed.

To provide a rough surface for the deposit silk threads were suspended in each of the tubes, a small glass bead being attached to the end of each one to keep it taut. The threads of tubes 3 and 4 were previously soaked for 5 minutes

in the extract. The threads from 1 and 3 were removed in 48 hours, the beads cut off, placed on a slide, gently moistened with xylol, and mounted in Canada balsam. The slide was then examined under the microscope, but no difference could be observed. The threads from 2 and 4 were removed at the end of 7 days and treated in a similar way. The thread from 2 showed no change, but even with the naked eye small grains like extremely fine sand could be seen clustered round the thread from tube 4. Under the microscope their granules had the typical appearance of tartar. Photo-micrographs from these two slides are shown in figures 1, 2, and 3. Thus we have shown that if the saliva and enzyme are present under favourable conditions, a deposit of a substance closely resembling tartar will occur in a short period of time.

A similar experiment was performed, using boiled enzyme extract. No sign of any substance could be seen on the thread.

Determination of the Nature of Organic Phosphate in Saliva.

Experiment No. 10.

In this experiment the phosphate was estimated by Brigg's method after precipitation of proteins by trichloracetic acid.

Twenty cc. of saliva were taken, and the protein removed with trichloracetic acid. Half the filtrate was boiled with 0·1 N sulphuric acid for 2 hours, and then the free phosphate estimated by Brigg's method. In the other half of the filtrate the free phosphate was estimated without previously hydrolyzing with acid, but the solution was made to 0·1 N sulphuric acid, to have both under identical conditions. The results are given below per 100 cc.

Before boiling with acid, 7·810 mg. expressed as phosphorus per 100 cc.
After boiling with acid, 9·10 mg. expressed as phosphorus per 100 cc.
Increase after boiling, 1·290 mg.

The experiment was repeated with another sample of saliva. Results:

Before boiling with acid, 8 mg. as phosphorus per 100 cc.
After boiling with acid, 10 mg. as phosphorus per 100 cc.
Increase after boiling, 2 mg.

From these results it appears as if there is present in saliva organic phosphate which can be hydrolysed by boiling with dilute acid, giving an increase in the amount of free phosphate present.

Experiment No. 11.

To determine the amount of sugar in saliva to 20 cc. of saliva after warming was added 3 cc. of dialyzed iron and 2 cc. of distilled water, and the solution filtered. Ten cc. of the filtrate were taken, made up to 20 cc., and the sugar estimated by McLean's method for blood sugar. Another 10 cc. of the filtrate

from the dialyzed iron treatment were boiled with 0·1 N sulphuric acid for an hour, and after neutralizing the acid present, the sugar was estimated as before. The results are given below:

		1.	2.
Before boiling with acid	=	0·0033% sugar	0·0041% sugar
After boiling with acid	=	0·0064% sugar	0·0077% sugar
Increase in sugar	=	0·0031% sugar	0·0036% sugar

The results show that the sugar content of the saliva was doubled by hydrolysis with acid.

Experiment No. 12.

An attempt was made to isolate the organic phosphate in saliva in a similar manner to that used by Kay for isolating glycerophosphate. 92 cc. of protein-free filtrate of saliva were diluted with an equal volume of water and $Ca(NO_3)_2$ added in excess, followed by ammonia till no more precipitate was obtained. In this way any free phosphate was removed from the mixture. The mixture was then filtered, and boiled to remove ammonia, neutralized to litmus, and excess of lead acetate (25%) added. On standing overnight a precipitate formed, and the mixture was centrifuged, the precipitate washed twice in water, and then suspended in warm water, and the lead removed by H_2S. The H_2S was removed from the filtrate with a current of air. The filtrate contained no free phosphorus; 10 cc. of the filtrate were hydrolysed with 0·1 N H_2SO_4 by boiling for 10 hours. The free phosphate was then estimated by Neumann's method, and the result was 0·00781 mg. P.

Another 10 cc. were digested with sulphuric and nitric acid, and the total phosphate estimated by Neumann's method, which gave 0·00792 mg. P. It thus appears as if the whole of the combined phosphate is liberated by boiling with acid.

On heating another portion of the filtrate with Fehling's solution a reduction occurred. On this result it was decided to test for the presence of sugar, but 20 cc. placed in a polarimeter tube gave no rotation.

On the assumption that the reducing power of the filtrate might be due to a sugar which would not give any rotation, 10 cc. of the filtrate were taken, and the sugar present estimated by McLean's blood sugar method. The result was 0·0008% sugar.

Another 10 cc. were taken and hydrolyzed with acid as in the previous experiment, and the percentage of sugar estimated. The result was 0·0016%.

This result is very interesting from the fact that both saliva and the filtrate prepared as described show a similar increase on hydrolysis with acid, i.e., the reducing power is doubled.

Experiment No. 13.

To determine whether human gum tissue contained pyrophosphatase, as described by Kay, an extract was made from gum tissue as above, and tubes were set up containing the extract and a solution of sodium pyrophosphate containing 2 mg. pyrophosphate per 10 cc. The pH was adjusted to 7·6, and the whole incubated for 24 hours. Any free phosphate present was estimated by Brigg's method. The results obtained showed that no hydrolysis due to enzyme action had taken place.

SUMMARY.

An active enzyme which is capable of hydrolysing glycerophosphoric ester is contained in the human gum tissue and in the oral tissue of the dog.

The saliva and blood serum contain phosphate-containing complexes (probably in the nature of phosphoric esters) capable of being hydrolysed by the enzyme.

The interaction of the enzyme on these phosphate-containing complexes results in the liberation and deposition of free inorganic phosphate.

Under favourable conditions the liberated inorganic phosphate will form concretions.

The action of the enzyme (liberated by trauma) on the saliva may thus give rise to salivary calculus.

The increase in free phosphate in saliva, accompanied by an increase in the copper-reducing power, on hydrolysis with 0·1 N acid points to the fact that the organic phosphate is in the nature of a phosphoric ester probably of the hexosephosphoric group.

Human oral gum tissue does not contain pyrophosphatase in any estimable quantity.

In conclusion, the author wishes to express his sincere thanks to Associate Professor W. J. Young, in whose department the work was carried out, for the great interest he has shown in the investigations, and for advice and help so generously given. He also wishes to acknowledge his indebtedness to the staff of the Biochemical Department for their ever-ready help, especially Miss Krieger, whose technical knowledge was at all times at the disposal of the author. Finally his thanks are due to Professor F. C. Wilkinson, of the Department of Dental Science, for his encouragement and much valued criticism.

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